UNIVERSIDADE FEDERAL DO PARANÁ

MARCEL KRUCHELSKI TSCHÁ

TAXON PULSE: UM MODELO PARA A DIVERSIFICAÇÃO GENÉTICA DE PEIXES EM BACIAS HIDROGRÁFICAS COSTEIRAS

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Tese apresentada à Coordenação do curso de Pós-Graduação em Ciências Biológicas - Zoologia, da Universidade Federal do Paraná, como requisito parcial para obtenção do título de Doutor em Ciências na área de Zoologia.

Orientador: Prof. Dr. Walter A. Boeger

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **MARCEL KRUCHELSKI TSCHA**, intitulada: **"TAXON PULSE: UM MODELO PARA A DIVERSIFICAÇÃO GENÉTICA DE PEIXES EM BACIAS HIDROGRÁFICAS COSTEIRAS"**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação.

Curitiba, 04 de Março de 2016.

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Ao constante sonho
de dignidade, justiça
e laicidade
dedico

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RESUMO

Bacias hidrográficas da margem atlântica da América do Sul engloba uma rica fauna de peixes. Os padrões filogenéticos e de distribuição das espécies sugerem associação com a história geológica dessa porção continental. Grandes eventos de mudanças climáticas também influenciaram a composição da ictiofauna nos tributários que deságuam no Oceano Atlântico. Sucessivas mudanças eustáticas moldaram a extensão das bacias hidrográficas litorâneas e promoveram episódios cíclicos de isolamento e contato entre populações de peixes. O que não foi diferente em um dos *hotspots* de biodiversidade da América Latina, a Bacia Litorânea do Paraná. Esta região possui uma fisiografia com feições geográficas de alto potencial para barreiras biogeográficas para espécies de menor vagilidade. Esse cenário é ideal para testar o modelo de diversificação de *Taxon Pulse* a nível genético. Esse modelo propõe que episódios oscilatórios de contração e expansão biótica sejam determinantes para a diversificação dos táxons. Portanto, o objetivo geral é testar um modelo de diversificação genética em escala microevolutiva utilizando marcadores moleculares, além de compreender como alteração do nível do mar afetam a estrutura genética e demográfica de comunidades de peixes. Ao todo, onze espécies de peixes dulcícolas e duas estuarinas, de bacias costeiras e de planaltos de altitude do estado do Paraná foram selecionadas. Análise Bayesiana de Skyline plot, mismatch distributions e testes estatísticos de neutralidade demonstraram aumento do tamanho efetivo populacional para as sete espécies de peixes, estuarinos e dulcícolas do litoral, condizente com períodos de baixo nível do mar. Por outro lado, esse mesmo padrão geral de história demográfica não foi observado para quatro espécies de planaltos de altitude. Em seguida, redes de haplótipos, testes de neutralidade e análises de história demográfica foram utilizados para três espécies de drenagens costeiras distribuídas em seis microbacias. Os resultados indicaram isolamento, contato secundário e expansão populacional, consistentes com a hipótese de isolamento e reconexão dos rios relacionados a mudanças no nível do mar. Por fim, padrões biogeográficos de seis espécies de peixes co-distribuídos foram testados utilizando reconstruções filogeográficas combinadas pelo algoritmo PACT. Foram encontrados sinais comuns intercalados de expansão e isolamento. A diversificação genética de peixes no estuário da Baía de Paranaguá e a distribuição das linhagens moldada pela influência das mudanças do nível do mar são compatíveis com os pressupostos da hipótese de *Taxon Pulse*. Pulsos de diversificação gerados por sucessivas fases de expansão e retração populacional são relacionados a dinâmica cíclica de quebra e formação de barreira geográfica causada pelas descida e elevação no nível oceânico.

ABSTRACT

Coastal watersheds in the Atlantic margin of South America comprise a rich fish fauna. Phylogenetic and distribution patterns of species suggest association with the geological history in this continental portion. Major events of climate changes also influenced the composition of the fish fauna in the tributaries that flow into the Atlantic Ocean. Successive eustatic changes shaped the extension of coastal watersheds and promoted cyclical episodes of isolation and contact between fish populations. It is not different in the coastal basin of Paraná, one of the biodiversity hotspots in Latin America. This region has a physiographic with geographic features that represents potential biogeographical barriers to species with lower vagility. This scenario is ideal to test the diversification model of Taxon Pulse at the genetic level. This model propose that oscillatory episodes of contraction and biotic expansion are crucial to diversification of taxa. Thus, the general goal is to test a model of genetic diversification in a microevolutionary scale using molecular markers, as well as understand how sea-level changes affect the genetic and demographic structure of fish communities. In total, eleven species of freshwater fishes and two estuarine species, from coastal basins and uplands in Paraná State were chosen. Bayesian skyline plot, mismatch distributions and statistical neutrality tests showed increases in effective population size for seven fish species, estuarine and freshwater from the coast, consistent with periods of low sea-level. On the other hand, the same general pattern of demographic history was not observed for four species from the uplands. Subsequently, haplotype networks, neutrality tests, and analyzes of demographic history were performed for three species of coastal drainages, distributed in six watersheds. Results suggested events of isolation, secondary contact, and population expansion, consistent with the hypothesis of rivers isolation and reconnection (expansion) related to sea-level changes. Finally, biogeographical patterns of six co-distributed fish species were tested, using phylogeographic reconstructions combined by the algorithm PACT. Common signs of interpolated expansion and isolation were found. The genetic diversification of fishes in the estuary of Paranaguá Bay and the distribution of lineages shaped by the influence of sea-level changes are compatible with the predictions of the hypothesis of Taxon Pulse. Pulses of diversification generated by successive periods of population expansion and contraction are related to cyclic and dynamic generation and breakdown of geographical barrier caused by the fall and rise of ocean levels.

PRÓLOGO

A compreensão dos processos envolvidos na diversificação de espécies e dos fatores que interferem na distribuição dos táxons em diferentes unidades geográficas é uma questão central nos estudos evolutivos. Dinâmica geológica, gradientes ecológicos e oscilações climáticas, em especial os movimentos eustáticos, fomentam a diversificação e biogeografia para grande parte dos peixes nas margens continentais. O passo inicial é desvendar como esses processos são determinantes na formação dos padrões de diversidade regionais. Para isso, torna-se essencial investigar os efeitos desses eventos históricos sobre a ictiofauna através de dados moleculares para múltiplas espécies co-distribuídas.

A presente tese integra história demográfica, estruturação populacional e filogeografia comparada de espécies de peixes a fim de examinar os processos de diversificação que levaram às atuais distribuições geográficas da ictiofauna em riachos costeiros. Visando subsequentes publicações em periódicos científicos, os três capítulos que compõem esta tese apresentam-se em língua inglesa e estão tematicamente assim divididos. O primeiro compara a história demográfica de cinco espécies de peixes de água doce e duas estuarinas que vivem na planície costeira com quatro espécies de peixes dos planaltos de altitude paranaenses, com o objetivo de revelar distintas histórias demográficas relacionadas aos níveis históricos de variação do nível do mar. O segundo capítulo tem como meta avaliar se a água salgada é de fato uma barreira para espécies de peixes dulcícolas e relacionar as variações do nível do mar com estruturação genética e história demográfica de três espécies em bacias hidrográficas litorâneas hoje isoladas. O último capítulo testa padrões biogeográficos de seis espécies de peixes co-distribuídas. A partir de reconstruções filogeográficas combinadas pelo algoritmo PACT (Phylogenetic Analysis for Comparing Trees), tem como objetivo examinar sinais comuns de expansão e isolamento das populações que seguem os preceitos da hipótese de *Taxon Pulse*, além de examinar as condições históricas de paleodrenagens locais.

INTRODUÇÃO GERAL

O número atual de espécies de peixes no mundo excede todas as projeções feitas há poucos anos. Estimava-se existir entre 31.500 (Berra, 2001) e 32.500 (Nelson, 2006) espécies de peixes, mas somente o número de nomes válidos atualmente já é superior a 33.715 (Eschmeyer, 2016). Nos ambientes de água doce encontram-se níveis de complexidade e instabilidade superiores quando comparados aos ambientes marinhos. Na água doce, contatos entre populações são mais limitados pela existência de barreiras geográficas e a história de vida dos peixes é, portanto, mais sujeita a fatores climáticos, geológicos ou bióticos.

A América do Sul, em especial sua margem continental atlântica é um componente biogeográfico importante caracterizado por grupos diversos de peixes viventes em suas bacias hidrográficas. A ictiofauna de água doce neotropical é riquíssima, com mais de quatro mil espécies conhecidas (Reis et al., 2003) e estimativas apontam para o dobro de espécies existentes (Vari & Malabarba, 1998). Sua grande diversidade se dá por fatores ecológicos e históricos como resultado de milhões de anos. No entanto, a biogeografia dos peixes ainda é pouco conhecida na América do Sul. A falta de dados de distribuição dos táxons, o escasso conhecimento de sistemática e da dinâmica geológica relacionada aos eventos cladogenéticos são ainda limitações para o entendimento dos processos históricos da fauna de água doce sulamericana (Vari & Weitzman, 1990).

As bacias atlântica e costeira da América do Sul, em sua maioria no Brasil são áreas de alto endemismo icitiofaunístico (e.g. Vari, 1988; Schaefer, 1997). Seus padrões filogenéticos nos dão indícios de uma relação de proximidade entre os rios que deságuam no Oceano Atlântico e seus propínquos rios de bacias dos planaltos de maior altitude. Esses padrões filogenéticos e de distribuição das espécies sugerem uma forte associação com a história geológica dessa porção continental desde o Cretáceo (Cobbold et al., 2001). Eventos de cladogênese estão possivelmente associados a processos vicariantes entre drenagens de terras altas do escudo cristalino e os tributários

do Oceano Atlântico (Ribeiro, 2006). As principais forças geológicas que atuam sobre a distribuição da ictiofauna nessa região são soerguimentos macrodômicos, rifteamentos, movimentos verticais de blocos e o recuo erosivo da costa, além da intensa atividade tectônica associada à separação dos continentes da África e da América do Sul que definiu a configuração do curso da maioria das bacias hidrográficas atuais (Almeida, 1976; Souza et al., 1996; Mohriak & Barros, 1990; Almeida & Carneiro, 1998; Thomaz-Filho et al., 2000; Riccomini et al., 2004).

Além dos processos geológicos, grandes eventos de mudanças climáticas também influenciaram a composição e distribuição da ictiofauna da drenagem atlântico costeira. A costa da América do Sul sofreu sucessivas transgressões e regressões marinhas durante o Quaternário (Pleistoceno e Holoceno) que desempenharam um papel importante na sua formação (Suguio et al., 1985). Alterações do nível do mar promoveram ciclos de isolamento e união de bacias costeiras e possivelmente influenciaram a diversificação da fauna aquática regional. Pelo menos três grandes ciclos de transgressões e regressões marítimas ocorreram desde o Pleistoceno no sudeste da costa brasileira (Suguio et al., 1985; Martin et al., 1996). Embora exista discrepância quanto suas datações mais recentes (ver Angulo et al., 2006), estima-se que áreas de água salgada se encontravam até 10 m acima do nível do mar atual há 123.000 anos atrás e 5,3 m há 5.100 anos (Suguio et al., 1985; Martin et al., 1996; Angulo & Lessa 1997; Martin, 2003). Esses dois eventos de transgressão marinha foram separados por um evento de regressão há 18.000 anos, associados ao último período glacial, quando o nível oceânico esteve há 130 m abaixo do atual na margem sudeste do Brasil (Kowsmann & Costa, 1974; Corrêa, 1996). April et al. (2013) destacam que ciclos de períodos glaciais do Pleistoceno desempenharam um papel preponderante na geração de diversidade de peixes.

Segundo Weitzman et al. (1988) a diversificação da fauna de peixes das bacias costeiras da América do Sul é relacionada às mudanças do nível do mar durante o Pleistoceno Superior. No entanto, características geológicas também foram fundamentais à ictiofauna local permitindo conexões entre as bacias dos

planaltos de altitude e rios da planície costeira brasileira (Ribeiro, 2006) e consequentemente fluxo gênico entre as populações.

A fisiografia ímpar da planície costeira paranaense, sua história geológica combinada a alterações climáticas do passado também parecem ter influenciado a formação das comunidades aquáticas. Esse conjunto, formado pelo oceano, bacias hidrográficas, baías e montanhas representam feições geográficas com alto potencial para barreiras biogeográficas de espécies com menor vagilidade, mesmo considerando a pequena escala geográfica da área. Estudos preliminares, de fato, demonstram que microbacias de planície costeiras próximas apresentam identidades taxonômicas e genéticas distintas (Pereira et al., 2013).

A análise do perfil genético de populações e o seu relacionamento revelam assinaturas tanto de processos atuais, tais como comportamento e migração, quanto de eventos históricos como vicariância e mudanças climáticas. Segundo Grant & Bowen (1998), altos índices de diversidade genética geralmente são indicativos de uma história evolutiva longa e estável, ou são resultado de contato secundário entre diferentes linhagens, enquanto baixa diversidade (tanto haplotípica quanto de nucleotídeos) são evidências de gargalos populacionais ou de eventos fundadores recentes associados a uma ou poucas linhagens.

Alguns padrões genéticos em escala de paisagem são quase sempre produtos de barreiras geográficas ao fluxo genético, originadas em períodos geológicos antigos, anteriores ou ao longo do Pleistoceno (Stepien et al., 2009). Barreiras geográficas podem ser responsáveis pelo acúmulo de diferenças genéticas entre as populações. Quando isoladas, as populações tendem a divergir por efeito de deriva genética, possivelmente acompanhada de pressões seletivas locais (Snoj et al., 2008). Portanto, a manutenção da diversidade genética é extremamente importante para que as populações possam responder às contínuas mudanças ambientais, evitando os efeitos negativos da deriva genética (Frankham et al., 2004).

A presente tese tem por objetivo geral analisar a importância de eventos de alteração do nível do mar e da fisiografia sobre a estrutura genética e

demográfica de comunidades da Bacia Litorânea do Paraná, a qual é especialmente importante por se localizar dentro de um dos *hotspots* de biodiversidade da América Latina. Este estudo visa também testar um modelo de diversificação genética de espécies de água doce da planície costeira brasileira utilizando marcadores moleculares aplicados a espécies de peixes representantes das comunidades analisadas.

A diversificação genética de peixes nos estuários e a distribuição das linhagens moldadas pela influência das mudanças do nível do mar parecem se encaixar nos pressupostos do modelo de *Taxon Pulse* (Erwin, 1979; 1981; 1985). Esse modelo ecológico prevê pulsos de diversificação a partir de sucessivas fases de expansão e retração populacional relacionadas à cíclica e dinâmica formação e quebra de barreiras geográficas. *Taxon Pulse* caracteriza-se por expansões bióticas amplas quando barreiras são quebradas e táxons dispersam para novos habitats, porventura mais adequados. Evolução geológica, operando em escalas de tempo mais longas que a evolução biológica, pode produzir barreiras, resultando em episódios de especiação vicariante e afetando a biota (Halas et al., 2005). *Taxon Pulse* assume episódios oscilatórios de expansão e retração populacional em um modelo unidirecional de diversificação (Liebherr & Hajek, 1990; Halas et al., 2005). Cada pulso de diversificação é iniciado por mudanças ecológicas, geológicas ou paleoclimáicas (Erwin, 1985) como por exemplo, as alterações no nível oceânico.

A principal hipótese é de que isolamentos e reconexões da fauna aquática de microbacias na planície costeira gerados pelas variações do nível do mar e pela fisiografia da Serra do Mar promoveram linhagens genéticas exclusivas. Essa hipótese assume que durante as subsequentes regressões marinhas a conexão entre as microbacias foi restabelecida, promovendo a mistura das linhagens antes isoladas e gerando um perfil genético altamente diversificado. Se aceita a hipótese de diversificação em pulso, parece pertinente associar os mesmos processos à diversificação taxonômica também encontrada nesses ambientes e que tem sido uma dúvida frequentemente levantada por ictiólogos trabalhando na região.

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CAPÍTULO I.

Sea-level variation influences the demographic history of estuarine and freshwater fishes of the coastal plain of Paraná, Brazil.

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Running title: Population history of coastal fish in Paraná

Abstract: Marine regressions and transgressions triggered by glaciations during the Quaternary have affected the diversification of the aquatic fauna on the Atlantic coast of South America. Cyclic sea-level changes have shaped the extension of coastal watersheds and thereby mediated connectivity and isolation of the affected fish populations. Nine freshwater and two estuarine/marine fish species of coastal basins and watersheds in highland plateaus of Paraná, Brazil, were screened for their mitochondrial haplotype diversity by sequencing either parts of the cytochrome b gene or the control region, respectively. The demographic history of each species was inferred by the Bayesian skyline plotting method, mismatch distribution analysis, and statistical neutrality tests. The demographic reconstruction analyses revealed the existence of a single pattern of *N*e variation. For the upland species no dramatic changes in the effective population size (*N*e) over time was inferred. In contrast, *N*e of all coastal plain species showed a conspicuous signal of population expansion over the last two hundred thousand years. These estimates correspond to periods of low sea-level (regressions) that were followed by rapid increases of the sea-level by more than 100 meters. The resulting reconnections and subsequent separations of the estuarine freshwater bodies supposedly promoted a substantial genetic diversification of the fish species.

Key words: effective population size, eustasy, Late Pleistocene, skyline plot.

Introduction

The Neotropical freshwater fish fauna comprises more than 4,000 known species (Reis et al., 2003), and its great diversity is expected to reflect millions of years of geological and ecological processes (Gery, 1969; Vari & Malabarba, 1998; Albert et al., 2011). Accordingly, it has been suggested that the biogeographic distribution pattern of the ostariophysan-dominated ichthyofauna of this broad zoogeographic region along the Eastern Brazilian coast was also shaped by the paleogeography of the watercourses and further constrained by the regional landscape and other ecological parameters (Albert et al., 2011). Today, the coastal plains of Brazil represent areas with a high proportion of endemic fish species (Vari, 1988; Bizerril, 1994; Schaefer, 1997) dwelling in streams and rivers that drain the eastern slopes of the Brazilian shield directly into the Atlantic Ocean (Abilhoa et al., 2011).

Processes triggered by climate changes during the Quaternary have strongly affected the distribution and demographic fluctuations of species in many regions of the world (Hewitt, 2000). The coastline of South America, for instance, was exposed to several marine transgressions and regressions during this period (Suguio et al., 1985; Martin et al., 1996; Angulo et al., 2006). In southeastern Brazil, the sea-level changes promoted at least three major cycles of marine regression and transgression during the interglacial periods of the Quaternary (Suguio et al., 1985; Martin et al., 1996), although there remain uncertainties in the precise dating (see Angulo et al., 2006). The sea-level was 10 m higher than today about 120 thousand years ago (kya) and about 5 m some 5.1 kya (Suguio et al., 1985; Martin et al., 1996; Angulo & Lessa, 1997). During a regression associated with the last glacial period ca. 18 kya the sea-level at the southeastern coast of Brazil was about 130 m below the present (Kowsmann & Costa, 1974; Correa, 1996). Supposedly, these cyclic sea-level changes caused cyclic isolation and reconnection of the coastal hydrographic basins.

Freshwater fish species confined to specific drainage systems represent an evolutionary conserved system to study distribution patterns reflecting continental and climate change effects of the past (Lévêque et al., 2008).

Accordingly, for Weitzman et al. (1988) the diversification of fish species in isolated watersheds of the South America coast very likely resulted from sea-level changes during the Late Pleistocene. The tectonic processes as well as the cyclic sea-level changes supposedly also affected the population structure of the fish fauna by facilitating periods with either isolation or reconnections between the river basins in highlands plateaus and the rivers of the coastal plain in Brazil (Ribeiro, 2006) that, accordingly, prevented or allowed for gene flow among populations.

The coastal lowlands of the state of Paraná, in southern Brazil, represent a biodiversity hotspot of Latin America (Mittermeier et al., 2005) with a significant proportion of endemic species of freshwater fish (Albert et al., 2011). As in other areas along coastal lowlands of Brazil, especially those associated with aquatic biodiversity hotspots, the Serra do Mar and its foothills to the west represent biogeographic barriers to freshwater fish species. This is particularly true for species with low tolerance to salinity variation and limited vagility. The Serra do Mar is an up to 1,800 meters high mountainous formation of crystalline shields (Almeida, 1976) that bears the head waters of all major rivers of the Paraná coastal plain. These river systems include many smaller watersheds, isolated by the foothills of this mountainous system and by the Antonina and Guaraqueçaba bays (both part of the Paranaguá bay system; see Fig. 1). Geographically isolated by the Serra do Mar to the west, south and north and by the ocean to the east the biodiversity of the watersheds in the coast plain of Paraná was most likely particularly exposed to the effects of historical sea-level changes. Hence, it is expected that the current diversity and the population histories during the Late Pleistocene of the freshwater or freshwater-dependent marine species should reflect eustasic changes.

This study addresses the demographic history of eleven coastal and highland plateau fish species of Paraná, Brazil, and targets the impact of historical eustatic sea-level changes on their population structure. We report changing effective population sizes of coastal fishes that are interpreted as responses to cyclic sea-level changes. We propose that these cyclic changes of isolation and reconnection of habitats were the major causes for the high

taxonomic and genetic diversity of the extant aquatic fauna of these coastal watersheds.

Materials and Methods

Experimental design and sampling

Eleven fish species from the hydrographic basins east and west of the Serra do Mar mountain range (Fig. 1) were selected that represented (i) marine species that are highly dependent of freshwater and estuarine habitats, (ii) freshwater species from spatially restricted basins of the coastal plains, and (iii) species from rivers west of the Serra do Mar that drain into the Iguaçu river. The demographic histories of the fish species of the first two groups may be affected by eustatic sea-level changes whereas those of the latter group are expected to be unaffected.

The marine species highly dependent of freshwater or estuarine environments (group i) include *Atherinella brasiliensis* (Quoy & Gaimard, 1825) (Atheriniformes: Atherinopsidae) and *Centropomus parallelus* Poey, 1860 (Perciformes: Centropomidae). Both species are common along the Brazilian coast (Figueiredo & Menezes, 1978; Rivas, 1986). The freshwater species from small river basins of the coastal plain (group ii) include *Characidium lanei* Travassos, 1967 (Characiformes: Crenuchidae), *Kronichthys lacerta* (Nichols, 1919) (Siluriformes: Loricariidae), *Mimagoniates microlepis* (Steindachner, 1877) (Characiformes: Characidae), *Scleromystax barbatus* (Quoy & Gaimard, 1824) (Siluriformes: Callichthyidae), and one undetermined species of *Rineloricaria* Bleeker, 1862 (Siluriformes: Loricariidae). Group iii includes the highland plateau species *Astyanax altiparanae* Garutti & Britski, 2000, *Astyanax bifasciatus* Garavello & Sampaio, 2010, *Mimagoniates* sp. (all three Characiformes: Characidae), and *Rhamdia quelen* (Quoy & Gaimard, 1824) (Siluriformes: Heptapteridae).

All fish were caught by either cast nets, fishing rod, or electro-fishing. All samples were collected under license number 10007 (Instituto Chico Mendes de

Conservação da Biodiversidade - ICMBio, Brazil). Muscle tissue samples were fixed in hypersaturated saline DMSO solution (Seutin et al., 1991). Whenever possible fin clips were collected instead of muscle samples in order to avoid that larger animals were sacrificed. Details of the sampling sites are shown in Figure 1.

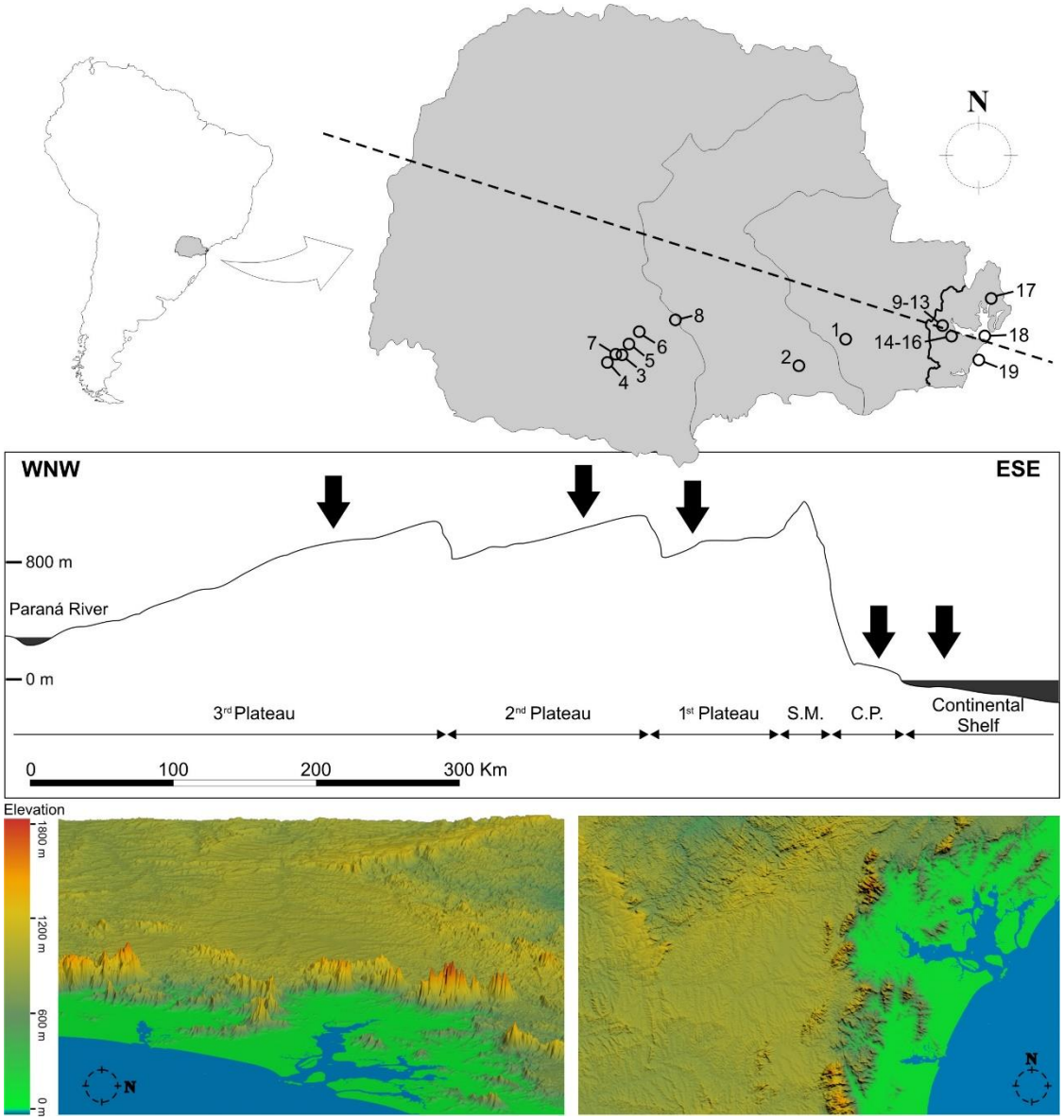

Figure 1. Sampling sites in highlands plateaus and coastal plain of Paraná State, Brazil. The numbered circles and arrows represent the sampling sites according Table I. Below, an orthographic projection (left) and a top view (right) illustrate the significant elevation differences between these areas. S.M.: Serra do Mar mountains, C.P.: Coastal plain.

Molecular procedures

Total genomic DNA was extracted with the DNA iPrep™ (Invitrogen) robot using the standard protocol of the iPrep™ ChargeSwitch® gDNA Tissue kit. The cytochrome b fragment (CytB) was amplified for all species except for *As. bifasciatus* using the primers GLUDG.L (Palumbi, 1996) and H16460 (Perdices & Doadrio, 2001). Since amplification of the targeted CytB fragment failed for *As. bifasciatus* part of the mitochondrial control region (D-loop) was amplified instead using the primers H16498 (Meyer et al., 1990) and L15774M (Prioli et al., 2002). CytB diversity of *Ce. parallelus* was studied earlier by Prodocimo et al. (2008). For this species CytB sequences were retrieved from GenBank.

DNA amplifications were performed in 25 µL PCR reactions containing 1 ngµl^{-1} of genomic DNA template, 1x buffer, 3 mM $MgCl_2$, 2.5 U Taq polymerase, 0.4 mM of each dNTP, and 2 nM of each primer. The PCR protocol consisted of an initial denaturation step of 4 min at 95 °C; followed by 35 cycles of 45 s at 94 °C, 45 s at 45 °C (60°C for the D-loop region), 45 s at 72 °C; and a final extension of 5 min at 72 °C. PCR success was controlled by agarose electrophoresis and subsequent ethidium bromide staining of the products. PCR products were purified with PEG 8000 (Amresco Inc., EUA). DNA sequencing reactions were carried out in 10 µl reaction mixes consisting of: 5 ng/µl template DNA, 0.5 µl Big Dye™ (Applied Biosystems Inc., Foster City, CA, U.S.A.), 0.2 µM of each primer, and 0.1 x reaction buffer. The final product was purified using Sephadex™ G-50 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Nucleotide sequences obtained for both strands were edited using Staden 1.6.0 (Staden, 1996), and subsequently aligned using the ClustalW (Thompson et al., 1994) algorithm. Basic sequence analyses such as estimating haplotype diversity (*h*) and nucleotide diversity (π) were done using Arlequin v3.5 (Excoffier & Lischer, 2010). Sequences are in GenBank (accession numbers: from KR423869 to KR424087).

Genetic analyses

The demographic histories (variation in the effective population size (*N*e)) for each species was reconstructed by the coalescent based Bayesian Skyline

Plot (BSP) method using the software BEAST v.1.6.1 and Tracer v.1.5 (Drummond & Rambaut, 2007). We implemented MCMC runs for 120 million generations sampled at each 1,000 trees, and 10% burn-in and strict molecular clock. The nucleotide substitution models were inferred for each species using JModelTest (Posada, 2008) and the corrected Akaike information criterion (AIC) (Table I). It is well known that mutation rates may vary substantially between genes and taxa (Nabholz et al., 2009). For the current dataset we choose 0.0076 substitutions/site/million years for CytB (Zardoya & Doadrio, 1999) and 0.0167 substitutions/site/million years for the *D-loop* region (Sivasundar et al., 2001). Demographic histories were also estimated by mismatch distribution (Rogers & Harpending, 1992) and neutrality tests according to Tajima (1989) and Fu (1997). Both were performed in Arlequin v.3.5 (Excoffier & Lischer, 2010). Genetic diversities indices of current theta (Θπ) and historical theta (Θw) were estimated by coalescent simulations in DnaSP v.5 (Librado & Rozas, 2009).

Results

A roughly 900 bp stretch of the mitochondrial cytochrome b (CytB) gene was successfully sequenced for nine fish species from coastal and upland watercourses of Paraná, Brazil. The CytB dataset was complemented by 89 sequences of *C. parallelus* taken from Prodocimo et al. (2008). For *As. bifasciatus* from the 3rd plateau a PCR amplification of the targeted CytB fragment were not available, most likely because the selected primer pairs were not suitable for this species, thus, a roughly 500-bp fragment of the mitochondrial control region (D-loop) was sequenced instead.

Fu's *Fs* and Tajima's *D* neutral tests were significant and negative indicating a recent demographic expansion of almost all species in coastal plain, however, Tajima's *D* was not significant only for *S. barbatus*, while Fu's *Fs* was not significant for *S. barbatus* and *Rineloricaria* sp. Species from uplands mostly were positive, but not significant for values of neutral tests. The average values of Θw (historical) were always lower than the average values of Θπ (current) in

lowlands and the opposite in uplands. However, 95% confidence interval for Θw included Θπ. Details on the number of observed mitochondrial haplotypes, haplotype diversity (*h*), nucleotide diversity (π), Tajima's *D*, Fu's *Fs*, and theta values are summarized in Table I.

The Bayesian skyline plot analyses revealed the existence of a single pattern of *N*e variation over the last two hundred thousand years for the targeted fish species. While the upland species sampled depicts no generalized shape of the historic variation of *N*e (Fig. 2A), a single pattern is conspicuous for all coastal species (Fig. 2B). The curves of demographic changes present a slight decline, a subsequent increase, and recent stabilization in the effective population size. However, presenting this same general curve pattern, the demographic reconstructions for *Rineloricaria* sp., and *S. barbatus*, are all characterized by a steep increase in *N*e by < 50 kya while for *At. brasiliensis*, *Ce. parallelus*, *Ch. lanei*, *K. lacerta*, and *M. microlepis,* the substantial increase in *N*e was postulated to start earlier, around 160-180 kya (Fig. 2B).

Mismatch distribution analyses also revealed similar pattern only for populations of fishes inhabiting coastal streams. The expected distribution of a population expansion is observed in all coastal fishes. Mismatch distribution of *Rineloricaria sp.* and *S. barbatus* share signal of possible secondary contact. On the other hand, there is no uniformity in the mismatch distributions for upland populations (Fig. 2).

Table I. Details on the sampling locations in each region. The mtDNA fragment, sample size, substitution model, diversity indices, haplotype number, values of neutrality, and values of theta for each species. (to be continued)

Site	Species	N	geographic coordinates (number of site in Figure 1)	mtDNA fragment	Substitution model	Haplotype diversity (h) / nucleotide diversity (π)	Haplotype number	Tajima's *D*	Fu's *FS*	current theta (Θi)	historical theta (W)
1st Plateau	*Mimagoniates* sp.	19	25°33'55"S 49°42'44"W (1)	CytB	TrN+G	0.105 ± 0.0920 / 0.000225 ± 0.000319	3	-1.511	0.021	0.310	0.309 (0,000/1,144)
2nd Plateau	*Astyanax altiparanae*	9	25°47'40"S 50°11'54"W (2)	CytB	TrN	0.6389 ± 0.1258 / 0.017457 ± 0.009788	3	1.680	9.448	15.717	15.593 (4.783/34.950)
	Rhamdia quelen	41	25°47'40"S 50°11'54"W (2)	CytB	TrN+G	0.8244 ± 0.0390 / 0.018773 ± 0.009499	13	0.873	7.104	15.711	15.960 (8.180/28.514)
3rd Plateau	*Astyanax bifasciatus*	62	25°41'54"S 51°59'30"W (3) 25°46'21"S 52°07'01"W (4) 25°39'11"S 51°53'60"W (5) 25°32'09"S 51°48'45"W (6) 25°43'15"S 52°00'20"W (7) 25°26'38"S 51°27'01"W (8)	D-loop	TrN+G	0.9270 ± 0.0206 / 0.050460 ± 0.024596	33	-0.5944	3.202	6.407	6.359 (3.194/10.860)
Coastal Plain	*Mimagoniates microlepis*	39	25°29'60"S 48°49'04"W (9) 25°30'50"S 48°50'34"W (10) 25°29'10"S 48°49'48"W (11) 25°30'11"S 48°51'25"W (12) 25°30'31"S 48°52'30"W (13)	CytB	TrN	0.9036 ± 0.0163 / 0.002930 ± 0.001727	19	-1.606*	-7.190*	2.418	2.372 (0.710/4.730)
	Rineloricaria sp.	56	25°31'35"S 48°45'03"W (14) 25°32'14"S 48°46'00"W (15) 25°33'24"S 48°48'05"W (16)	CytB	HKY+G	0.8055 ± 0.0233 / 0.004218 ± 0.002318	11	-1.686*	-3.828	1.549	1.544 (0.435/3.265)

Scleromystax barbatus	68	25°29'60"S 48°49'04"W (9) 25°29'10"S 48°49'48"W (11) 25°30'11"S 48°51'25"W (12) 25°30'31"S 48°52'30"W (13)	CytB	HKY+G	0.8572 ± 0.0189 / 0.005387 ± 0.002958	15	-0.770	-3.849	2.312	2.287 (0.835/4.385)
Characidium lanei	19	25°29'10"S 48°49'48"W (11)	CytB	HKY	0.7895 ± 0.0995 / 0.002942 ± 0.001813	11	-2.434*	-4.454*	2.829	2.813 (0.858/6.008)
Kronichthys lacerta	15	25°12'48"S 48°17'52"W (17)	CytB	GTR	0.8936 ± 0.0258 / 0.002557 ± 0.001582	6	-1.882*	-11.796*	2.303	2.297 (0.835/4.385)
Atherinella brasiliensis	18	25°33'33"S 48°21'43"W (18)	CytB	HKY + G	0.9250 ± 0.0469 / 0.002217 ± 0.001505	10	-1.614*	-5.704*	1.830	1.828 (0.301/4.219)
*Centropomus parallelus***	89	25°41'S 48°38"W (19)	CytB	TrN	0.6481 ± 0.0609 / 0.002968 ± 0.001938	37	-2.762*	-27.829*	1.675	1.654 (0.395/3.360)

* indicates significant values ($p < 0.05$ to Tajima's *D*; $p < 0.02$ to Fu's *Fs*).

** - data taken from Prodocimo et al. (2008).

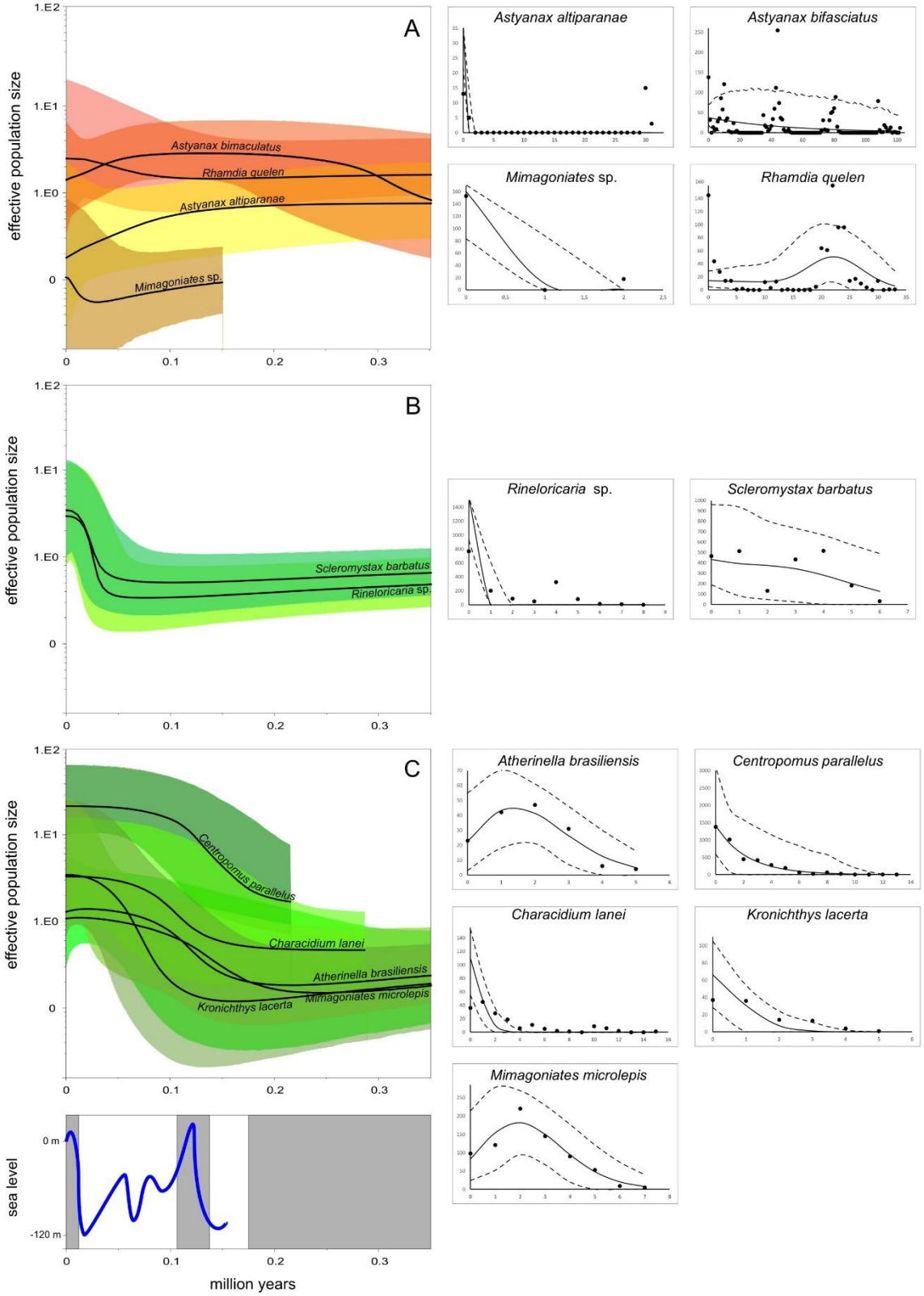

Figure 2. The past demographic by the Bayesian skyline plot for species in coastal plain (A and B) and highlands plateaus (C). The box under represents sea-level in the same time scale in the region and the gray bands show interglacial periods (D). Mismatch distributions are shown beside their skyline plot groups.

Discussion

The shared pattern of the demographic curves strongly suggests the influence of regional environmental processes shaping the genetic profile of the populations of all species population sampled on the coastal plain streams. On the other side, no generalized pattern was shared for the reconstruction of the demographic histories for the species collected in the neighbor highland plateaus. To the west of the Serra do Mar mountain range, the highland populations had variations in *N*e apparently not related with the same regional events that generated the demographic growth pattern on coastal species. In the uplands, each species seems to have its specific history, probably associated to intrinsic biological characteristics of each, but the environmental driving forces associated to their respective demography is unclear.

The timing of the substantial increases of *N*e in the skyline plots (Fig. 2) roughly correlates to sea-level changes (eustasy). The putative beginning of the *Ne* increase in the set of coastal populations apparently correlates with the lowering of sea-level subsequent to sea-level maxima. Glacioeustasy is driven by the formation and melting of polar ice sheets during the glacial-interglacial cycles. During the last 200,000 years, at least three cycles have been postulated that affected the eastern Brazilian lowlands (Suguio et al., 1985; Martin et al., 1996). These date estimates for the increase in *N*e deduced for the coastal fish populations coincide with a period of relatively low sea-level bordered by two large transgressions (Fig. 2C) (Kowsmann & Costa, 1974; Suguio et al., 1985; Correa, 1996; Martin et al., 1996; Angulo & Lessa, 1997; Angulo et al., 2006).

Variations in sea-level during the Pleistocene putatively promoted the expansion of the coastal freshwater basins during low sea-levels which were flooded subsequently with salt water during sea-level transgressions. With the increase in the area of distribution and expansion of the previously isolated populations of freshwater fishes likely fused into a large panmictic group, promoting the observed increase in *Ne.* Further support to the interpretation of population expansion in the coastal populations are the mismatch analyses (Fig. 2) and the negative values of Tajima's *D* and Fu's *FS*. Values of historical theta

and current theta, however, were not sufficiently significant to provide differences in coalescence histories between the coastal and highland regions. Recent stabilization observed in the reconstructed demographic curves for the coastal populations likely relates with the recent gradual fragmentation of the ancestral river basin associated with the sea transgression, reducing habitat and population size.

Similar scenarios were also reported for Characiformes, a primary freshwater fish group, in the Amazon basin (Hubert & Renno, 2006), for the cichlid fauna of Lake Tanganyika in East Africa (Nevado et al., 2013), and for some fish species in the Great Lakes of North America (April et al., 2013). Impact of sea-level changes on fish genetic history has been also reported for the silverside, *Odontesthes perugiae* Evermann & Kendall, 1906 species complex (Atherinopsinae) from seven lakes and one river in Southern Brazil (Beheregaray et al., 2002).

Although similar in the general curve pattern, there is clearly an asynchrony in the variation of *N*e reconstructed for the species of the coastal plain of Paraná. While the begin of the populational increase was dated at < 50 kya for *Rineloricaria* sp., and *S. barbatus*, for *At. brasiliensis*, *Ce. parallelus*, *Ch. lanei*, *K. lacerta*, and *M. microlepis* the increase of *Ne* started at of ca. 160-180 kya. This asynchrony apparently reflects at least one of two things: 1) That the single rate of evolution used are inadequate for all species and the apparent asynchrony is an artifact; or 2) there are indeed biological differences in the response to environmental changes between the two sets of species studied.

However, as suggested by Grant et al. (2012), demographic simulations, such as Bayesian skyline plots, can only infer the last most significant populational genetic changes. Thus, even assuming adequate date estimates the method would not be capable of detecting the effect of earlier glacial cycles over the effective population sizes of *Rineloricaria* sp. and *S. barbatus*. In contrast, the Bayesian skyline plots suggest that the *N*e of *At. brasiliensis*, *Ch. lanei, Ce. parallelus, K. lacerta*, and *M. microlepis* would be little affected by the last glacial cycle directly. These species, contrary to *Rineloricaria* sp. and *S. barbatus*, were strongly influenced by the penultimate reduction in sea-level due to the effects of

glacioeustasy, while the influence of comparatively lower sea-level fluctuations during the most recent 100,000 years (Fig. 2) were not significant or detected. Curiously, despite sharing similar demographic patterns, *At. brasiliensis* and *Ce. parallelus* are mostly marine species but reproduce in estuaries and freshwater environments. These biological characteristics of both species are likely responsible by the demographic pattern shared with truly freshwater species.

The results of the current study add to a growing body of examples pointing on the importance of changing sea-levels on the demographic history of coastal freshwater species. Moreover, these environmental changes likely influence other level of complexity, such as biodiversity, population genetic profile and community structure. In future studies, it might be particularly interesting to assess how sea-level changes affect these distinct levels of biologically complexity of coastal fish species.

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CAPÍTULO II.

Connection and isolation of watersheds affecting the diversification and distribution of genetic variability of coastal freshwater fishes.

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Running title: Watersheds dynamic and a genetic history of coastal freshwater fishes

Abstract: The Atlantic coast of South America is characterized by a great diversity and endemism of fishes. Past eustatic changes that promoted cycles of isolation, expansion, and connection of coastal watersheds are considered putative drivers of genetic differentiation and phylogenetic diversity. Recent eustatic movements should have left signs of impact on the demographic history and local distribution patterns of freshwater fishes. This study addressed the phylogeography and demographic history of two siluriform (*Scleromystax barbatus*, *Rineloricaria* sp.) and one characiform (*Mimagoniates microlepis*) fish species from the coastal plain of Paraná State, Brazil, around the Paranaguá Bay, an area that is bordered by the ocean to the east, and the Serra do Mar mountain range and a wide continental shelf to the west. Nucleotide sequence data of > 800 bp of the mitochondrial cytochrome b gene supports the genetic differentiation between populations of the three species among all studied watersheds. Haplotype networks indicate different histories with scenarios of secondary contact, population expansion, and isolation of the studied populations. Neutrality tests and the reconstructed patterns of demographic history in mismatch distributions were also consistent with secondary contact in northern basins and population expansion mainly to western populations. The results are consistent with the hypothesis that recurrent reconnection and isolation of streams associated with eustatic changes strongly influences the current pattern of diversity and distribution of freshwater fishes in this coastal hydrographic system.

Key words: drainage systems, glaciation, Pleistocene, seal-level changes.

Introduction

Roughly 40% of all fish species occur in freshwater (Dudgeon et al., 2006) and, on a global scale, the neotropical freshwater ichthyofauna is considered the most diverse (Schaefer, 1998; Reis et al., 2003; Albert et al., 2011). Approximately 70% of neotropical freshwater fish species occur exclusively in the streams of the Atlantic forest in the Brazillian coast (Abilhoa et al., 2011). In particular, the drainage systems of eastern South America represent regions with high level of endemism of fish species (e.g. Vari, 1988; Bizerril, 1994; Schaefer, 1997). Communities that are confined in drainage systems provide ideal conditions for studies that may reflect the past traits in their populations due to the environmental changes (Lévêque et al., 2008).

The unique physiography of the Atlantic coast in the Paraná State, Brazil, is an ideal model to investigate diversification processes triggered by geological history and paleoclimatology. According to Gery (1969), the region is part of South-East Brazilian ichthyofaunal province and the Southeastern Mata Atlantica ecoregion, as defined by Abell et al. (2008). These coastal plains are surrounded by the foothills of the Serra do Mar mountain range formed by uplifts during the Cenozoic (Almeida, 1976), and all streams and rivers are running West-East and drain into the Atlantic Ocean. The coastal plains of Paraná are recognized as a distinct area in terms of their ichthyofauna with a predominance of small-sized fish species such as tetras (Characidae) and armored catfish (Callichthyidae and Loricariidae) (Abilhoa et al., 2011).

Erosive processes were, according to Ribeiro (2006), decisive in determining the composition of fish communities in the coastal watersheds of eastern of Brazil. However, extensive sea-level changes, responsible by marine transgressions and regressions since the Pleistocene, strongly influenced the exchange and diversification in the region. The sea-level exceeded the current level about 123 kya and 5.1 kya (Suguio et al., 1985; Martin et al., 1996; Angulo & Lessa, 1997; Martin, 2003) but was roughly 130 m below the present sea-level some 18 kya (Kowsmann & Costa, 1974; Correa, 1996). Such cyclic eustatic movements are presently accepted as drivers of genetic diversity, demographic

history, and regional distribution patterns of freshwater fishes worldwide (e.g. Roxo et al., 2012; April et al., 2013; Chakona et al., 2013a; Pereira et al., 2013; Roxo et al., 2014; Bruno et al., 2015; Tschá et al., unpublished).

Weitzman et al. (1988) were the first to propose the diversification of fishes in the presently isolated watersheds of the South American coast associated to glaciation cycles and sea-level changes during the Late Pleistocene. Subsequently, studies documented that eustatic changes and geographic barriers influenced the radiation of *Odontesthes* species, the silverside fish, in southern lakes of Brazil (Beheregaray et al., 2002) and affected the patterns of dispersion and vicariance of *Hoplias malabaricus*, a widely distributed species in the coastal lakes of Brazil (Pereira et al., 2013). Marine retreats during glacial periods of the Pleistocene provided opportunities for dispersal of freshwater fishes among currently isolated watersheds along the Brazilian coast and acted as a major factor structuring the recent divergence of *Hollandichthys multifasciatus* (Eigenmann & Norris, 1900), an endemic fish of drainages in southeastern coast of Brazil (Thomaz et al., 2015). Additionaly, Baggio et al. (unpublished) revealed distinct patterns of genetic variation of estuarine fishes (*Atherinella brasiliensis* (Quoy & Gaimard, 1825) influenced by eustatic movements that matched features of continental shelf along the Brazilian coast. Finally, a recent study of Tschá et al. (unpublished) documented three different kinds of demographic history signals when studying eleven fish species of the coastal lowlands and highlands plateaus in Paraná, Brazil. These authors tied a unique and common pattern observed in fish populations in the coastal lowlands to sea-level changes. In general, the species from lowland watersheds present clear demographic expansion correspond that overlapped with periods of sea-level increase of the sea-level by more than 100 meters.

Under this putative scenario, cycles of marine regression and transgression should allow connection (with expansion) and isolation of fish populations from local watersheds. Expansion should result in the mixture of genetic profiles evolved in allopatry during the preceding transgression among neighboring watersheds. In the special case of the watersheds surrounding the Paranaguá Bay, which are limited by the Serra do Mar, transgressions with

associated reduction in habitat should result in bottleneck events, that should have been imprinted in the genome. This cyclic pattern of expansion and isolation, with the associated consequences, should be observed at several levels of biological complexity, including in population genetics, as in the present study. However, under the present scenario, this model of genetic diversification and distribution can only be observed if the marine and estuarine environments (i.e. water salinity higher than freshwater) truly represent a barrier for extensive dispersion of the species.

Thus, this study has two major objectives to evaluate: 1) The significance of salt and brackish water as a barrier for the freshwater fish species, and 2) the existence of common genetic signals in populations of this fish assemblage linked to the last cycle of regression/transgression.

If saltwater indeed acted as a barrier to gene flow among freshwater fish species of the coastal river basins in Southern Brazil, it is expected that even species in geographically close watersheds should have evolved unique genetic structure, while still showing signatures of a common gene-pool. Among the consequences of the last eustatic movement, a demographic signal of bottleneck should precede a populational increase overlapping the period of marine transgression. In order to test these predictions and the model of genetic diversification, this study focuses on three freshwater fish species distributed in coastal watersheds in the coastal plain of Paraná State, Brazil.

Materials and Methods

Sampling and molecular procedures

Three fish species were targeted in this study, representing distinct biology and ecology. *Mimagoniates microlepis* (Steindachner, 1877) (Characiformes: Characidae), *Scleromystax barbatus* (Quoy & Gaimard, 1824) (Siluriformes: Callichthyidae), and an unidentified species of *Rineloricaria* Bleeker, 1862 (Siluriformes: Loricariidae) were caught by cast nets or electro-fishing under license number 10007 (Instituto Chico Mendes de Conservação da

Biodiversidade - ICMBio, Brazil). These species inhabit the same clearwater coastal streams but differ substantially in their life history. *Mimagoniates microlepis* is widely distributed (Weitzman et al., 1988), but prefers waters with vegetal covering (Mazzoni & Iglesias, 2002). Furthermore, it has a higher vagility and a rather low salinity tolerance (Freire et al., 2008). The catfish *Scleromystax barbatus* prefers lentic sites. A diurnal foraging pattern in backwaters of shallow streams is characteristic for this species (Aranha et al., 1998; Golçalves & Cestari, 2013). In contrast, *Rineloricaria* species occur in various habitats, including streams or larger rivers with strong water rapids but also lakes with shallow water and sandy soil (Reis & Cardoso, 2001). However, little is known specifically about the biology and taxonomy of the target species of *Rineloricaria* in the coast of Paraná State.

Samples were collected from six river sub-basins around the bay of Antonina and bay of Laranjeiras, part of the complex of the Paranaguá Bay, Paraná, Brazil. The river sub-basins were: Sagrado River, Nhundiaquara River, Cacatu River, Tagaçaba River, Serra Negra River, and Guaraqueçaba River. As there are no obvious migration barriers within each sub-basin, all specimens of a species within each sub-basin were considered as belonging to the same panmictic population. Accordingly, the fish from different sampling sites within each sub-basin were pooled in the analyses. Three of the selected sub-basins are located around the Antonina bay (western sites 1, 2, and 3) and another three around the Laranjeiras bay (northern sites 4, 5, and 6). Details of the sampling sites are illustrated in Figure 1, and the respective geographic coordinates are provided in Table 1.

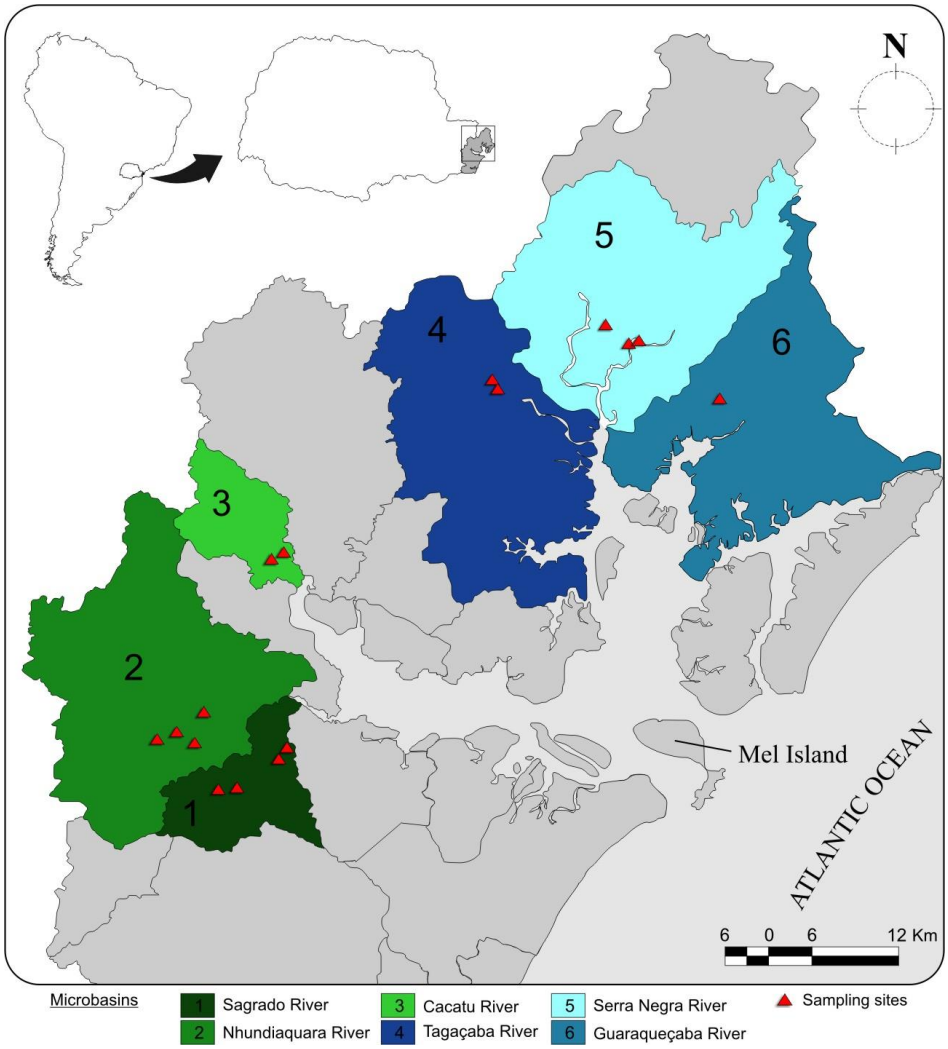

Figure 1. Sampling sites for the three species in the six targeted watersheds around the bay of Antonina (sites 1, 2, and 3) and bay of Laranjeiras (sites 4, 5, and 6), both minor extensions of the great bay of Paranaguá, Paraná, Brazil. The western basins are depicted in green and the northern basins in blue colors (for details see Table 1).

Muscle tissue samples were fixed in hypersaturated saline DMSO solution (Seutin et al., 1991), while the remaining parts of the sampled fish were preserved in 95% ethanol, and subsequently registered into the collection of Museu de História Natural do Capão da Imbuia. Total genomic DNA was extracted using the EZ-DNA kit (Applied Biosystems) and the DNA iPrep™ (Invitrogen) robot with the standard protocol of the iPrep™ ChargeSwitch gDNA Tissue kit ™ (Invitrogen). A fragment of > 800 bp of the mitochondrial cytochrome b gene (CytB) was amplified for all specimens using the primers GLUDG.L (Palumbi, 1996) and H16460 (Perdices & Doadrio, 2001). PCR amplifications were

performed in 25 µL containing 1 ng/µl genomic DNA template, 1x buffer, 3 mM $MgCl_2$, 2.5 U Taq polymerase, 0.4 mM of each dNTP, and 2 nM of each primer. The PCR protocol consisted of an initial denaturation step of 4 min at 95 °C; followed by 35 cycles of 45 s at 94 °C, 45 s at 45 °C, 45 s at 72 °C; and a final extension of 5 min at 72 °C. PCR success was controlled by agarose gel electrophoresis, and the positive PCR products were purified with PEG 8000 (Amresco). DNA sequencing of the PCR products was carried out in 10 µl reaction mixes consisting of: 5 ng/µl template DNA, 0.5 µl Big Dye™ (Applied Biosystems), 0.2 µM of primer, and 0.1 x reaction buffer. The sequencing reactions were purified using SephadexTM G-50™ (GE Healthcare Bio-Sciences AB). Nucleotide sequences obtained for both strands were edited using Staden v.1.6.0 (Staden, 1996), and subsequently aligned using ClustalW (Thompson et al., 1994) as implemented in BioEdit v.7.0.5 (Hall, 1999). Nucleotide sequences are deposited in GenBank.

Table 1. Sampling sites, rivers correspondent to each watershed, geographical coordinates and municipality.

Site	Hidrographical basin	river	GPS coordinate	Locality
1	Sagrado	Sagrado	S 25° 33' 23.93" W 48° 48' 04.97"	Morretes, PR, Brazil
			S 25° 32' 14.29" W 48° 46' 00.08"	Morretes, PR, Brazil
			S 25° 31' 34.64" W 48° 45' 03.20"	Morretes, PR, Brazil
		do Meio	S 25° 33' 32.78" W 48° 48' 55.93"	Morretes, PR, Brazil
2	Nhundiaquara	do Pinto	S 25° 30' 50.30" W 48° 50' 34.27"	Morretes, PR, Brazil
		Marumbi	S 25° 30' 31.49" W 48° 52' 30.26"	Morretes, PR, Brazil
			S 25° 30' 11.15" W 48° 51' 25.38"	Morretes, PR, Brazil
			S 25° 29' 10.46" W 48° 49' 48.08"	Morretes, PR, Brazil
3	Cacatu	Cacatu	S 25° 19' 25.03" W 48° 45' 06.99"	Antonina, PR, Brazil
		do Nunes	S 25° 20' 19.23" W 48° 47' 04.60"	Antonina, PR, Brazil
4	Tagaçaba	Tagaçaba	S 25° 11' 38.74" W 48° 31' 17.81"	Antonina, PR, Brazil
		Capivari	S 25° 11' 03.42" W 48° 31' 22.82"	Antonina, PR, Brazil
5	Serra Negra	Pederneiras	S 25° 08' 34.83" W 48° 23' 38.08"	Guaraqueçaba, PR, Brazil
		Bananal	S 25° 10' 41.51" W 48° 22' 51.43"	Guaraqueçaba, PR, Brazil
			S 25° 10' 31.25" W 48° 22' 18.63"	Guaraqueçaba, PR, Brazil
6	Guaraqueçaba	Morato	S 25° 12' 48.87" W 48° 17' 52.62"	Guaraqueçaba, PR, Brazil

Genetic analysis

The estimation of genetic diversity indices as well as the analysis of molecular variance (AMOVA) were done using Arlequin v.3.5 (Excoffier &

Lischer, 2010). Overall and pairwise F_{ST} values were calculated for all sub-basins as well as for the pooled western (1-3) and northern (4-6) sub-basins using a permutation of 1,000 iterations. Significance levels for multiple pairwise comparisons were corrected with the Bonferroni method (Rice, 1989). To test isolation by distance were also performed Mantel tests (Mantel, 1967) in Arlequin v.3.5 (Excoffier & Lischer, 2010), using F_{ST} values. For this purpose, geographic distance was measured as straight-lines between the estuaries of each watershed using Google Earth™.

To reconstruct the demographic history for each sub-basin, haplotype networks were constructed, mismatch distribution (Rogers & Harpending, 1992) were plotted, and neutrality tests according to Tajima (1989) and Fu (1997) were calculated. Tajima's D and Fu's F_S statistics and mismatch distribution were produced in Arlequin v.3.5 (Excoffier & Lischer, 2010). Haplotype networks were derived in TCS v.1.21 (Clement et al., 2000) using the parsimony criterion. Demographic histories were also reconstructed by Bayesian Skyline Plots using BEAST v.1.7.5 (Drummond et al., 2012) and Tracer v.1.5 (Rambaut & Drummond, 2009). The best fitting nucleotide substitution model was determined using jModelTest v.0.1 (Posada, 2008) using the corrected Akaike Information Criterion (AICc). Bayesian Inference analyses were conducted in four independent MCMC runs for 100 million generations sampled at each 1,000 trees. The first 10% of trees were discarded as burn-in. To provide an approximate time frame for coalescent reconstruction we used the commonly accepted substitution rates of 0.0076 substitutions/site/million years for CytB mtDNA of freshwater fishes (Zardoya & Doadrio, 1999).

Haplotype accumulation curves were conducted in the statistical program R v.3.1.2 (R Development Core Team) using the package spider (Brown et al., 2012). It was performed to evaluate sampling effort and to compare the haplotype diversity among populations from northern sub-basins (that flow into Laranjeiras Bay) and western sub-basins (that flow into Antonina Bay). This process calculated the mean accumulation of haplotypes and its standard deviation through 1000 random permutations, similar to the method that generate rarefaction curves (Gotelli & Colwell, 2001).

Results

For all three targeted fish species, a substantial number of mitochondrial haplotypes were detected. Haplotype diversity (*h*) and nucleotide diversity (π) were in the same order of magnitude for *M. microlepis* (n = 173; 43 haplotypes, *h* = 0.904 ± 0.016; π = 0.003 ± 0.002), *S. barbatus* (n = 181; 47 haplotypes; *h* = 0.875 ± 0.0145; π = 0.006 ± 0.003), and *Rineloricaria* sp. (n = 205; 41 haplotypes, *h* = 0.806 ± 0.023; π = 0.004 ± 0.002). Most haplotypes were only found in one locality, but in *M. microlepis* four, *S. barbatus* three, and *Rineloricaria* sp. five haplotypes were found in more than one sampling site. Details on the haplotypes and their distribution are listed in Table 2.

Table 2. Summary of sample size, haplotype numbers, haplotype diversity (*h*), nucleotinde diversity (π), Tajima's *D* and Fu's *Fs* estimated from cytochrome b mtDNA fragments.

species	site	sample size (n)	haplotype numbers	haplotype diversity (*h*)	nucleotide diversity (π)	Tajimas's *D*	Fu's *Fs*
Mimagoniates microlepis	1	53	10	0.7293 ± 0.0513	0.002036 ± 0.001307	-1.14458	-2.02697
	2	39	15	0.8677 ± 0.0384	0.002460 ± 0.001529	**-1.60641**	**-7.18958**
	3	21	4	0.6238 ± 0.0899	0.000924 ± 0.000756	0.10336	-0.28559
	4	24	8	0.8442 ± 0.0441	0.001618 ± 0.001123	-0.33110	-2.75995
	5	13	2	0.5385 ± 0.0602	0.001722 ± 0.001223	2.12145	3.78917
	6	23	10	0.8617 ± 0.0489	0.003135 ± 0.001900	-0.59425	**-2.32260**
	total	173	43	0.9036 ± 0.0163	0.002930 ± 0.001727	-2.4192	-1.79925
Rineloricaria sp.	1	56	11	0.5006 ± 0.0771	0.001506 ± 0.001014	**-1.68622**	**-3.82751**
	2	78	14	0.8275 ± 0.0221	0.002311 ± 0.001409	-0.45889	-3.27654
	3	20	6	0.5158 ± 0.1316	0.001231 ± 0.000903	**-2.09572**	-1.55231
	4	10	5	0.8667 ± 0.0714	0.002157 ± 0.001467	-0.43167	-0.36263
	5	20	7	0.7947 ± 0.0598	0.007992 ± 0.004319	1.49078	3.74126
	6	21	6	0.6381 ± 0.0978	0.003079 ± 0.001852	-1.36143	1.18500
	total	205	41	0.8055 ± 0.0233	0.004218 ± 0.002318	-0.75719	-0.68212
Scleromystax barbatus	1	45	15	0.6384 ± 0.0825	0.001715 ± 0.001189	**-2.18013**	**-10.84172**
	2	68	14	0.7954 ± 0.0286	0.002813 ± 0.001729	-0.76978	-3.84906
	3	22	6	0.7186 ± 0.0845	0.001621 ± 0.001168	-1.29478	-1.26182
	4	8	2	0.2500 ± 0.1802	0.000305 ± 0.000432	-1.05482	-0.18197
	5	28	5	0.5291 ± 0.1054	0.001436 ± 0.001059	**-2.17023**	-1.25654
	6	14	8	0.8681 ± 0.0684	0.005856 ± 0.003416	-0.81700	-0.54232
	total	185	47	0.8747 ± 0.0148	0.005998 ± 0.003248	-1.38112	-2.98890

*bold numbers of Tajima's *D* and Fu's *Fs* indicate significance at *p* values < 0.05.

The haplotype networks for the three species illustrate the low number of more frequent and widespread haplotypes, and the few instances of haplotypes shared between the sampled sub-basins (Fig. 2). In *S. barbatus* there are two well-defined haplogroups; the first one restricted to the western sub-basins and the second comprising haplotypes only detected in the northern sub-basins. In *Rineloricaria* sp. and *M. microlepis* there are also haplogroups found at the northern sites; however, the respective haplotypes occur also in some watersheds in the west.

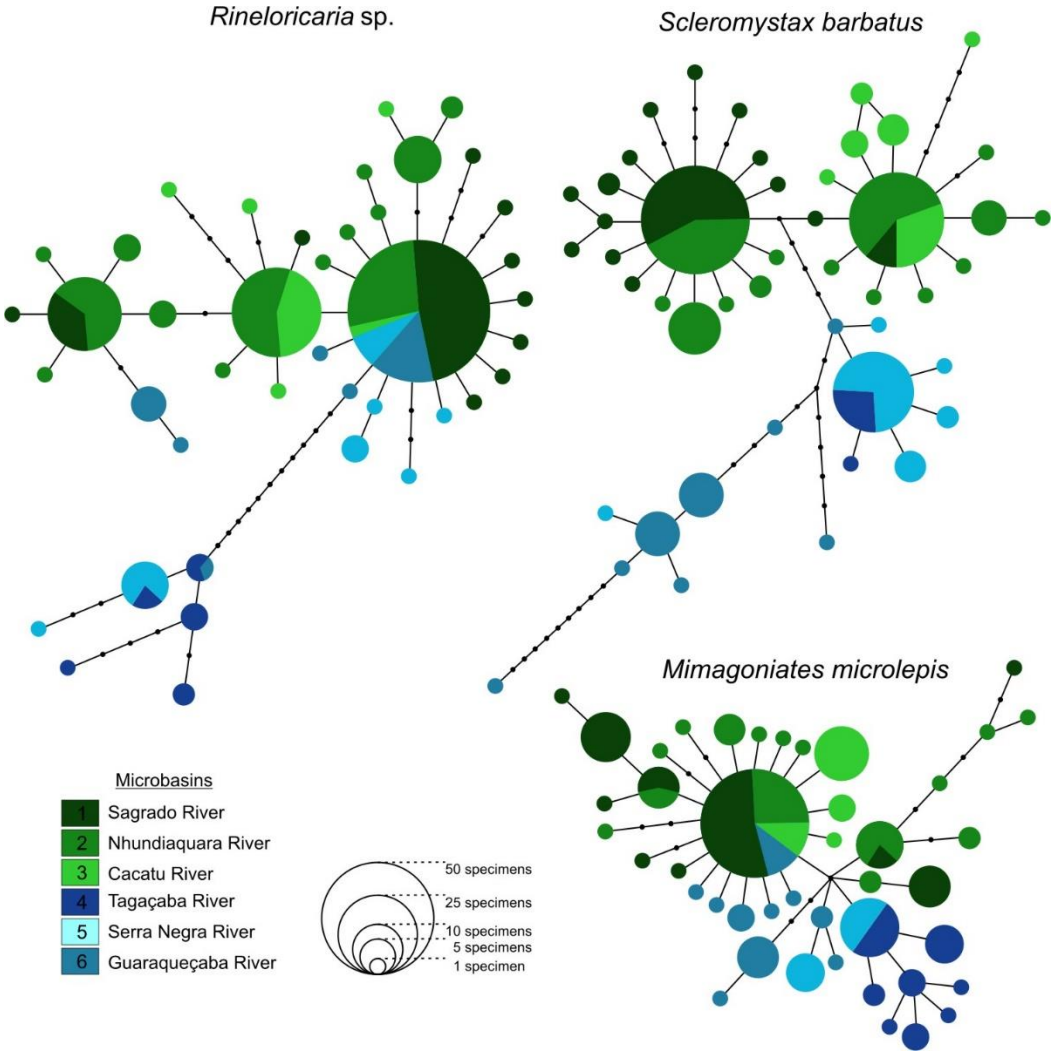

Figure 2. Haplotype networks for *Mimagoniates microlepis*, *Rineloricaria* sp., and *Scleromystax barbatus* in the six target watersheds.

The AMOVA revealed higher genetic variation within than between populations in the six watersheds for *M. microlepis* and *Rineloricaria* sp. (60.4%, $p < 0.01$ and 48.0%, $p < 0.01$, respectively). For both species just a relatively low

fraction of 18.6% (p < 0.01) for *M. microlepis* and 24.3% (p < 0.01) for *Rineloricaria* sp. of variation related to northern vs western groups. However, a somewhat different result was obtained for *S. barbatus*, in which 59.0% (p < 0.01) of the variation was between the western and northern groups, and only 23.3% (p < 0.01) was due to within population variation. Furthermore, variation between populations in the six watersheds was higher than within populations. Pairwise F_{ST} values indicated significant genetic differentiation between almost all populations for the three species, except for the Tagaçaba sub-basin and Serra Negra sub-basin populations of *S. barbatus* (Table 3). Mantel tests provided statistical support only for isolation by distance in *S. barbatus* (79.5%, p = 0.01), but these were not significant for *M. microlepis* (27.5%, p = 0.14) and *Rineloricaria* sp. (8.6%, p = 0.32).

Table 3. Estimated F_{ST} values between populations for each species.

	Mimagoniates microlepis			Global F_{ST}: 0.3959, p<0.001	
Locality	1	2	3	4	5
2	**0.08144**				
3	**0.18112**	**0.21364**			
4	**0.53401**	**0.46641**	**0.67519**		
5	**0.44737**	**0.35909**	**0.63471**	**0.33397**	
6	**0.21844**	**0.15448**	**0.31480**	**0.45083**	**0.31310**
	Rineloricaria sp.			Global F_{ST}: 0.51965 p<0.001	
Locality	1	2	3	4	5
2	**0.08798**				
3	**0.24856**	**0.13614**			
4	**0.88969**	**0.84863**	**0.89900**		
5	**0.40337**	**0.41474**	**0.37003**	**0.43025**	
6	**0.06673**	**0.07435**	**0.18548**	**0.80502**	**0.23170**
	Scleromystax barbatus			Global F_{ST}: 0.76716 p<0.001	
Locality	1	2	3	4	5
2	**0.17685**				
3	**0.64527**	**0.31518**			
4	**0.82167**	**0.72713**	**0.86960**		
5	**0.81160**	**0.73881**	**0.84886**	0.02504	
6	**0.78713**	**0.74646**	**0.77795**	**0.57424**	**0.63611**

* bold numbers have significant p values.

The mismatch distribution analyses revealed unimodal curves in all fish populations from the Tagaçaba sub-basin to the Sagrado sub-basin. Although few pairwise differences were observed to *S. barbatus* in Tagaçaba sub-basin. The historical demographic reconstruction for *Rineloricaria* sp. and *S. barbatus* demonstrate populations from the Guaraqueçaba and Serra Negra sub-basins with bimodal curves. In these sub-basins *M. microlepis* present characteristic of unimodal curves (Fig. 3).

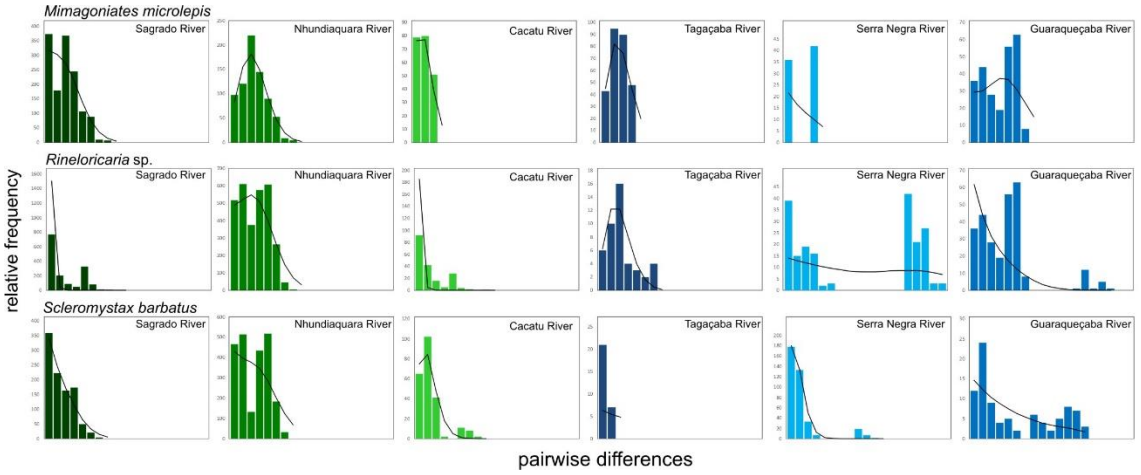

Figure 3. Mismatch distribution for *Mimagoniates microlepis*, *Rineloricaria* sp., and *Scleromystax barbatus*. Colors match the watersheds depicted in Figure 1.

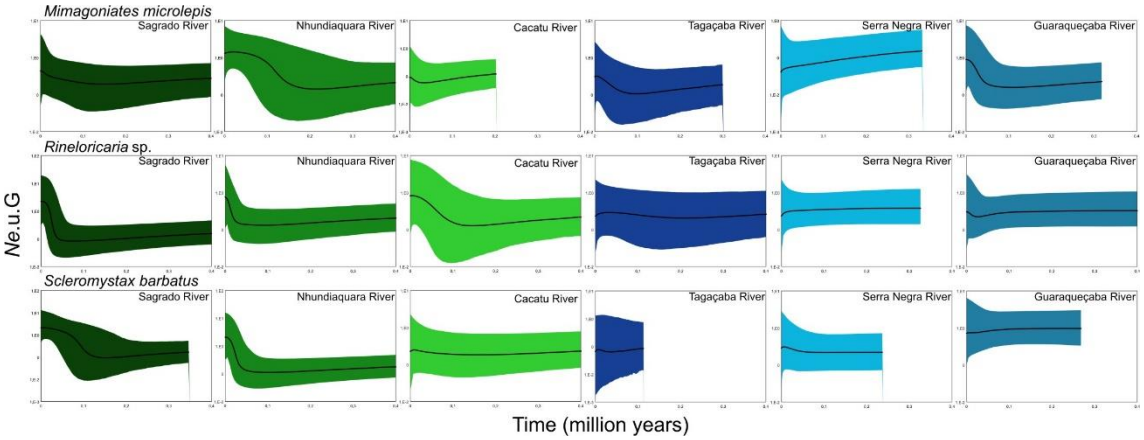

Figure 4. Demographic histories of populations of *Mimagoniates microlepis*, *Rineloricaria* sp. and *Scleromystax barbatus* reconstructed by Bayesian Skyline Plots. Thick lines represent means and colored areas the 95% confidence distribution of the effective population size. Colors match the watersheds depicted in Figure 1. All different graphs have the same x- and y-axis scales.

The Bayesian Skyline Plots indicate for all three species recent significant increases of Ne for the populations in the western sub-basins. For *M. microlepis* and *Rineloricaria* sp. a similar pattern was observed for the Tagaçaba and

Guaraqueçaba sub-basins. In the other northern sub-basins, however, changes in Ne were less pronounced showing either a slight increase or even a decrease in Ne (Fig. 4). Tajima's *D* and Fu's *FS* were negative for all species in all sampled sub-basins, but there was largely no statistical support for these estimates (Table 2).

Haplotype accumulation curves revealed that both groups of watersheds showed distinct levels of diversity at given sampling efforts (Fig. 5). It is clear that there are significant differences in their contributions to overall diversity estimates. The western group of watersheds captured the largest diversity in terms of haplotypes in all species, although, it was revealed that the CytB diversity have not been fully sampled in the western group, as indicated by the steep slopes of lines.

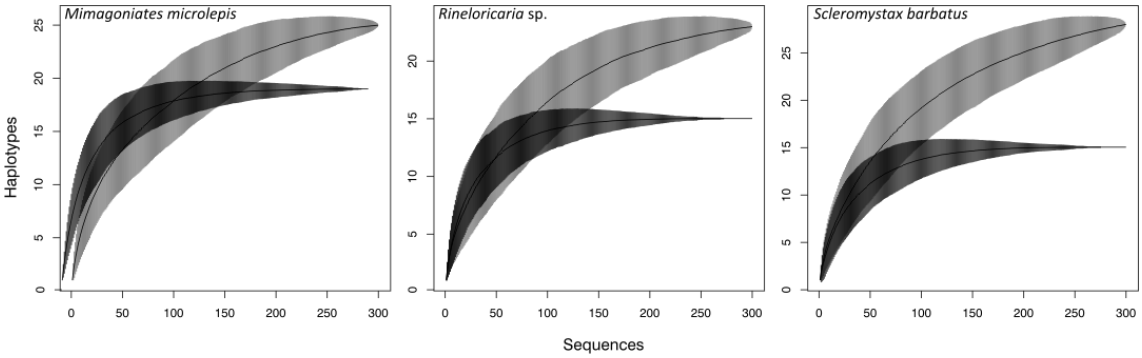

Figure 5. Haplotype accumulation curves for *Mimagoniates microlepis*, *Rineloricaria* sp. and *Scleromystax barbatus*. Standard deviation in black lines indicates sampling curve for northern watersheds, and standard deviation in gray lines represents sampling curve for western watersheds.

Discussion

The physiography of the coastal plain of the state of Paraná, Brazil, especially in the region around the Paranaguá Bay, represents an area that was particularly affected by extensive sea-level changes caused by marine transgressions and regressions during the Pleistocene. This area of the coastal plain is rich in small watersheds that are presently isolated by the branches of the Paranaguá Bay and the foothills of the Serra do Mar.

Under this geological scenario, eustatic changes promoted cycles of isolation and reconnection of these watersheds (Suguio et al., 1985; Martin et al., 1996) and consequently, of their freshwater fauna. We suggest that these recurrent cycles promoted diversification and may have influenced the regional richness of the freshwater fish fauna. However, our results also suggest that intrinsic characteristics of the species studied and regional episodic events promoted genetic exchange between watersheds and resulted in a complex molecular signal.

That the marine environment indeed represents a significant barrier for the species studied is evidenced by the strong genetic differentiation among populations of the characiform, *M. microlepis*, and the siluriforms, *S. barbatus* and *Rineloricaria* sp.*,* inhabiting distinct watersheds draining into the Antonina Bay and Laranjeiras Bay. Data available on historical sea-levels, strongly indicate that the fragmentation and isolation of these populations were maximum at the sea-level maximum, at 8 ± 2 m and 4.8 ± 0.5 m above present level about 123,000 and 5,100 years ago (Martin et al., 1996; Martin, 2003). Thus, the observed genetic differentiation may be interpreted as the result of the interaction between the geologic features of the region and the most recent transgression of sea-level associated with the end of the last glaciation period.

Indeed, recurrent changes in sea-level have been suggested to shape the evolution and diversification, and putatively affected the level of endemism of coastal fish populations (Chakona et al., 2013a; Pereira et al., 2013, Roxo et al., 2014; Baggio et al., unpublished). While during glacial periods, low sea-levels resulted in the confluence of rivers within geomorphological sections of the Eastern Brazilian coast, during interglacial periods, watersheds gradually isolated from each other by the rising sea-level with saltwater posing a geographic barrier for local freshwater fishes. We should, thus, expect to detect genetic signals associated with the last cycle of expansion (low sea-level) and isolation (high sea-level). With expansion, mixture of older haplotypes should be detectable while more recent haplotypes should present higher endemicity since they likely originated in allopatry due to the fragmentation of the collective older watershed. The proximity of the Serra do Mar mountain range to the Paranaguá Bay during

high sea-levels should result in signals of multiple bottlenecks (for distinct watersheds) followed by expansion, as a result of allopatric diversification within the fragments.

The topologies of the overall haplotype networks of the three target fish species (Fig. 2) are consistent with the scenario of bottleneck (single most abundant and central haplotypes) followed by secondary expansion (star-shaped pattern), as it should be expected with subsequent population isolation and reduction of the area of distribution. This pattern is especially conspicuous in the regions of the haplotype networks associated with watersheds located in the southwest margins of the Paranaguá Bay (Sagrado, Nhundiaquara, and Cacatu sub-basins). Further, especially for *Rineloricaria* sp. and *S. barbatus,* the existence of multiple central haplotypes with their respective satellite haplotypes is consistent with fragmentation into multiple watersheds and genetic drift followed by allopatric diversification, as indicated by the many endemic satellite haplotypes.

Population expansions, a consequence of allopatric diversification of haplotypes, are suggested also by the reconstructed patterns of demographic history inferred by the unimodal distribution in mismatch analyses, especially in western populations, (Fig. 3). Mismatch plots suggest secondary contact for populations of *S. barbatus* and *Rineloricaria* sp. from Guaraqueçaba and Serra Negra sub-basins (Fig. 3). The Bayesian Skyline Plot reconstructions also support population expansion more pronounced in the western populations (Fig. 4). Values of Tajima's *D* and Fu's *FS* were not significant and thus, are inconclusive (Table 2).

The results above clearly point to differences in the demographic histories of populations from the western and northern watersheds of the Paranguá Bay. While the signals of recent isolations of previously large populations linked to bottleneck followed by subsequent expansion is clear for western populations, the same pattern is not observed in the northern watersheds. There are two possible general explanations for these differences. 1) That there are fundamental differences in the pattern generated for these two groups watersheds, likely associated to the geomorphology of the areas that hindered

the establishment of the pattern of diversification predicted from the oscillation in sea-level; or 2) That the same expected diversification pattern occurred equally in both regions but the populations from the northern watersheds have lost the genetic signal of these events.

Haplotype accumulation curves (Fig. 5) indicate that the haplotype richness of northern populations are indeed smaller for all species studied herein, independent of sampling density. Haplogroups for the populations of Siluriformes (*Rineloricaria* sp. and *S. barbatus*) of the northern watersheds (Fig. 2) are composed almost exclusively by unique haplotypes, do not depict a clear star-like shape, and shows many indications of absent or extinct (missing intermediate) haplotypes. A unique haplogroup is also observed for the northern populations of *M. microlepis.* Further, general mismatch analyses (Fig. 3) indicate expansion for most northern population, as in the case of the western populations but many watersheds depict signals of secondary contact (for both species of Siluriformes). However, in Bayesian Skyline plots (Fig. 4), expansion is not as evident in the studied species from the northern watersheds.

However, there is no reasonable explanation to ascertain that the pattern of diversification expected from sea-level variations should be absent from northern watersheds in the Paranaguá Bay. It is, thus, proposed that differences in the diversification patterns likely reflect more recent processes, which could mask the genetic signals corresponding to the influence of sea-levels by promoting, as suggested by the haplotrees and mismatch analysis, local extinction and secondary dispersion of haplotypes among the northern watersheds. In some way, this hypothesis incorporates both alternative hypotheses propose to provide explanation to the differences encountered between the northern and the western watersheds.

Once again, the haplotrees provide some insights on the events that could have taken place and determined the differences observed between the northern and western watersheds. In all watersheds, the coexistence of haplotypes in populations genetically structured might be related to two process. First, sharing of more recent halotypes could be associated to current processes of dispersal and occasional weather conditions, such as the heavy precipitation events.

Second, sharing of older (central) haplotypes appear to be related to the spatial relationships of paleodrainages linked with the expansions and connection of presently isolated watersheds during periods of low sea-level.

Years with strong El Niño show extreme rainfalls, far above the historical mean in the Paraná Coast (Vanhoni & Mendonça, 2008; Pscheidt & Grimm, 2009), and may cause catastrophic and sporadic flooding in the region (see Abilhoa et al., 2011). In such years, large pluviometric volumes promote flooding that connect freshwater bodies. The large river discharge generates flushes into the bay freshwater species and creates unusually extensive freshwater plumes in the river mouth, allowing dispersion of species between neighboring river systems. Accordingly, Chakona et al. (2013a; 2013b) discussed if biotic range expansion of fishes in streams could have been related to connections during rain periods and concluded that sympatric occurrence of very divergent lineages is facilitated by rare events of intermittent wet connections resulting in secondary contact through dispersion. For the studied fish species such a scenario might seem unlikely since, in general, Characiformes and Siluriformes are primary freshwater fish species with apparent weak tolerance to even slight salinity levels. However, there are evidences that *M. microlepis* can sustain periods of exposition to marine salinity, maintaining their weight muscle stable for more than one hour (Freire et al., 2008) and, thus, should present greater ability to disperse between neighboring watersheds. This is likely associated with differences observed between the silurifom species and *M. microlepis. Mimagoniates microlepis* is also benthopelagic - as opposed to demersal as *Rineloricaria* sp. and *S. barbatus* (see Froese & Pauly, 2015) which likely favours dispersal between neigboring watersheds, in the less dense freshwater plumes. Survival of the siluriform species are, for the same reasons above, expected to be smaller during periods of high river flow, something that is consistent with the signals of haplotype extinctions for these species.

Marine regression records and bathymetric data are clues about the past connection of watersheds in the region. Expansion of regional watersheds formed a wide freshwater drainage system thousands of years ago. The connection facilitated contact between fish stocks of originally isolated drainages and

expansion of these populations into adjacent drainages. This process may have an important significant role in the diversification of fishes in coastal streams (e.g. Montoya-Burgos, 2003; Ribeiro 2006; Roxo et al., 2012, Dias et al., 2014; Roxo et al., 2014; Bruno et al., 2015; Thomaz et al., 2015). Bathymetric data of Paraná coast (for details see Angulo et al., 2006, Lamour et al., 2007) suggest two past regions of confluence of rivers draining into the Paranaguá bay. Despite anthropogenic impact with port dredging activities, there is one drainage channel from the bay of Antonina and another one from the bay of Laranjeiras that come together west of the Mel Island and bypass the island into the Atlantic Ocean (Fig. 6). This morphological paleochannel system indicated by bathymetric data is congruent to the genetic pattern found in the coastal plain. The pattern of haplogroups differentiated between western and northern populations, principally in *S. barbatus*, reflects the bifurcated geography in the bay and is related to the connectivity of ancient rivers. Haplogroups from western populations are related to the Antonina Bay and rivers that likely ran together in this region, while haplogroups related to northern populations are associated to the river branches on Laranjeiras Bay.

The largest continental shelf in South America is on the Paraná coast, with a distance of roughly 230 km from the shoreline to the continental slope (Mahiques et al., 2010). Therefore, periods of low sea-level during the glacial maximum may have been ideal for the confluence of river basins in deltas or estuaries in regions of the continental shelf (e.g. Suguio et al., 1985). The current hydrographic systems that are isolated by the saltwater formed a distinct scenario in the past. A connected system of river channels and valleys, today submerse, were connected in during low sea-level and allowed freshwater fish migrations and gene flow on the entire coastal plain. Similarly, Chakona et al., (2013a) hypothesized that confluence of adjacent rivers in the coast of South Africa during periods of lower sea-levels could have facilitated dispersal of genetically differentiated lineages, a scenario very similar to the one presented here for the coast of Paraná.

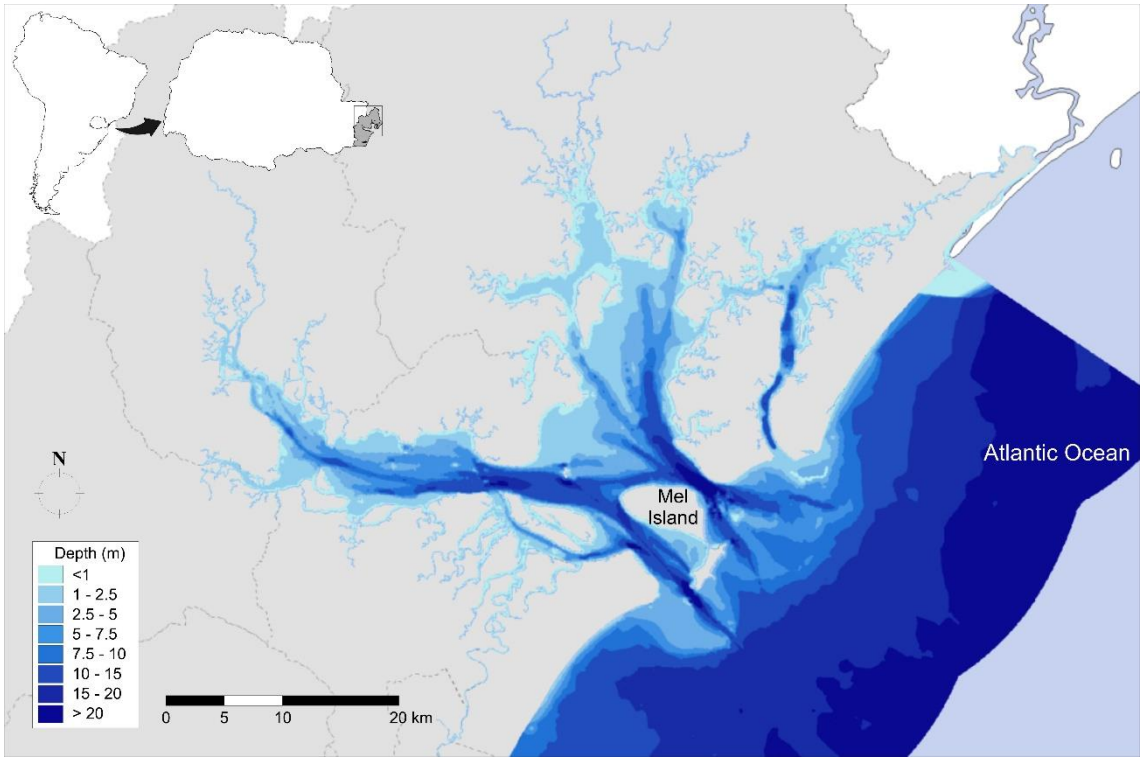

Figure 6. Bathymety in Paranaguá Bay, Brazil. Image font: IGIA, 2010.

Our results are consistent with signatures of isolation with eventual secondary contact, and population expansion. Therefore, we propose a model of genetic diversification of freshwater fish species by oscillatory episodes. The cyclic pattern observed in this study, with population expansion and contact associated with subsequent retraction and isolation strongly resembles the evolutionary dynamic known as taxon pulse hypothesis. Pulses of diversification were described by Erwin (1979; 1981; 1985) as repeated periods of contraction and biotic dispersion of taxa beginning in a central area of origin. According to this hypothesis, species generated by allopatric speciation could become widespread after overcoming barriers or after the breakdown of these barriers in repetitive episodes. Marine water acted as a barrier that isolated freshwater populations while sea-level regression, thousands of years later, allowed the genetic mixture among populations through the expansion of fish species previously isolated. This process is clearly a phenomenon of interest to biologists. Studies of historical biogeography in the region may reveal genetic diversity patterns of fish populations which, we predict, will comply with the assumptions of taxon pulse. Molecular dating is essential to infer if the mixing of lineages is

associated with periods of sea retreat and genetic divergences with periods of transgression.

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CAPÍTULO III.

Oscillation in phylogeography of coastal freshwater fishes: setting the stage for diversification by Taxon Pulse.

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Running title: pulse diversification of coastal fishes

Abstract

Aim To investigate the historical biogeography of freshwater fishes in a dynamic system of coastal drainages and test the hypothesis of genetic diversification by Taxon Pulse. To correlate signals of expansion and isolation in fish populations to sea-level changes and paleodrainages.

Location Watersheds in the coastal plain of Paraná State, southern Brazil.

Methods Phylogenetic inferences and algorithm of phylogenetic analysis for comparing trees (PACT) were used to reveal the shared history of six species of freshwater fishes. Final area cladograms were mapped to reveal geographical patterns associated to isolation or dispersal events. Bathymetric data and GIS methods were used to reconstruct paleodrainages during low sea-level periods. Regression analysis were performed to assess the relation between lineage-richness and the involved events. Curves of extant lineages accumulated through time were conducted in the statistical packages of software R.

Results The final area cladogram resulted in a tree with 169 terminal units and 64 shared nodes. The mapping of ancestral areas revealed multiple introduction of freshwater fishes at the Paranaguá Bay from northern watersheds. Four nodes were associated to isolation and fifteen to biotic expansion displayed in an interpolated pattern of events. Curves of extant lineages accumulated through time exposed periods of high accumulation of lineages interpolated with periors of stasis at least for two species. There was a positive relation between lineage-richness and the isolation/expansion events involved in each watershed. Predicted paleodrainages suggest that connection among rivers in the estuary occurred with only approx. 10 m of sea-level retreat.

Main conclusion The history of genetic diversification of the freshwater fish species in coastal drainages strongly correlates with alternating episodes of expansion and isolation associated with sea-level fluctuation and is compatible with the predictions of the hypothesis of Taxon Pulse.

Keywords

Coastal plain, paleodrainages, PACT, seal level changes, shared history.

Introduction

The present organization of continental watersheds in East South America was defined since the opening of the South Atlantic Ocean, approx. 95 million years ago (Granot & Dyment, 2015). Presently, around 93% of rivers in the mainland South America drain into the ocean and harbor a rich and distinctive ichthyofauna. Vicariant events generated by the division of drainages are significant correlated with fish diversification in the region (Lundberg et al., 1998). However, the composition and diversity of freshwater fishes in this continent is strongly associated to additional processes such as marine incursions, uplifting of mountain ranges, and complex histories of exchanging connections between drainage systems owing to headwater capture (Lovejoy et al., 1998; Malabarba, 1998; Montoya-Burgos, 2003; Hubert & Renno, 2006; Lovejoy et al., 2006; Ribeiro, 2006; Albert et al., 2011).

The interaction between faunal evolution and dynamic geological and environmental changes in the coastal river systems of Brazil is noteworthy. The life history of fishes in streams in the Atlantic rainforest are strongly influenced by climatic, geologic, or biotic factors, both on regional and local scales. A large number of barriers (i.e. saltwater and physiographic barriers) creating isolated areas result in frequent allopatric speciation events. Populations in small rivers exhibit reduced effective population size, enhancing the effects of genetic drift (Wright, 1940). The freshwater fish biota that lives in dynamic systems with variation in habitat stability are thought to be characterized by elevated rates of extinction and speciation (Bloom et al., 2013).

The coast of Paraná State, southern Brazil, is part of the Ecoregion known as Southeastern Mata Atlantica (Abell et al., 2008). This area consists in a coastal plain surrounded by foothills and several rivers that emerge on the eastern slope and flow into the Atlantic Ocean. The continental shelf extends about 230 km in the region (Mahiques et al., 2010). The relief of the marine floor is a clue about paleochannels that could have formed a large and connected freshwater system during the last maximum glacial, a period when the sea was below the current level (e.g. Dias et al., 2014; Bruno et al., 2015; Thomaz et al., 2015). The

watersheds in this coastal lowland are inhabited by a distinct and diverse ichthyofauna, characterized by catfishes, tetras, and other small fishes (Abilhoa et al., 2011). The evolution of fish populations in these regions is strongly associated with ancient geological events and geographic features in this continental portion. However, sea-level changes in the end of the Pleistocene also seem to have a strong influence on demographic histories and, hence, on regional rates of speciation (Tschá et al. unpublished).

A general model of historical biogeography demystified the perspective that all general nodes in an area cladogram refer to episodes of isolation due to vicariance and that expansions do not produce general patterns (Brooks, 2005). This model is based on the Taxon Pulse hypothesis (Erwin, 1979; 1981; 1985). Taxon Pulse proposes that episodes of vicariance and dispersion events, presenting periods of contraction and biotic expansion, have preponderant influence on the process of diversification at any level (i.e. genetic, speciation). Erwin's hypothesis shares many ideas with Taxon Cycle proposed by Wilson (1959; 1961) for island populations and based on the ideas of Darlington (1943). Erwin (1979) proposes the model of Taxon Pulse initially to explain the diversity and geographic patterns of carabid beetles in mainland. This hypothesis assumes oscillatory episodes of biotic expansion and vicariance, and encompasses a unidirectional model of diversification that occurs in relatively longer periods of time (Liebherr & Hajek, 1990; Halas et al., 2005). Erwin (1979) asserted that many clades disperse from a center area of origin and become widespread after the breakdown of barriers that previously produced vicariant processes by isolation among taxa of multiple clades (eg. Erwin & Adis, 1982). Each pulse begins with major ecological, geological, or paleoclimatic changes, for instance, with changes in global climate (Erwin, 1985) and variations in the ocean level.

Marine transgressions and regressions since the Late Pleistocene promoted cycles of isolation and reconnection of watersheds in the target coastal land. Saltwater was above the current sea-level at 123 kya and 5.1 kya (Angulo & Lessa, 1997; Suguio et al., 1985; Martin et al., 1996), and 130 m below the current level at 18 kya (Kowsmann & Costa, 1974; Correa, 1996). We believe

that this is the ideal scenario for pulse diversification as suggested by Erwin (1979). For many freshwater species, saltwater acts as an almost impermeable barrier. During marine incursions, it promotes isolation of freshwater populations in watersheds in the coast plain, thus facilitating differentiation of species and populations (Baggio, unpublished; Tschá et al., unpublished). The dissolution of such barriers, with subsequent sea retreat, promotes reconnection of watersheds, allowing new episodes of biotic expansion. Cycles of isolation and connection of rivers putatively set the stage for repeated episodes of range expansions and contractions, which fits the Taxon Pulse predictions. In fact, there are many evidences that fish diversification in isolated coastal drainages of south America was strongly dependent of sea-level changes during the Late Pleistocene (Weitzman et al., 1988) and the genetic diversity of coastal fishes is recently associated to eustatic movements (e.g. Roxo et al., 2012; Pereira et al., 2013; Roxo et al., 2014; Bruno et al., 2015; Baggio, unpublished; Tschá et al., unpublished).

Since the modern evolutionary synthesis (Mayr, 1942; 1947; Dobzhansky, 1951), macroevolutionary phenomena of speciation are regarded as the result of microevolutionary process of ecologically based divergent selection. This assertion suggests that events of expansion and isolation should be observed at several levels of biological complexity, including the microevolutionary scale of population genetics. We assume that no single fish population contains the complete information of an area history, or even about its own particular history. So, integrating information from each fish species/population and clades allows the reconstruction of the coevolutionary history of life in the target area.

Therefore, this study is the first to apply PACT [Phylogenetic Analysis for Comparing Trees] (Wojcicki & Brooks, 2004; 2005) to investigate the historical phylogeography of multiple freshwater fish species. Phylogeographic patterns of six species were examined in order to evaluate their diversification in a dynamic system of coastal drainages. This study also correlates sea-level changes in the region and historical conditions of paleodrainages to genetic signals of expansion and isolation in fish populations.

Materials and Methods

Sampling

We selected six freshwater fish species inhabiting thirteen clearwater rivers/streams in seven watersheds around the Paranaguá Bay, Paraná, Brazil. The main river in each sub-basins gives its name to the target watershed. In clockwise according to Figure 1: Sagrado river, Nhundiaquara river, Cacatu river, Cachoeira river, Tagaçaba river, Serra Negra river, and Guaraqueçaba river. The target species were chosen because they are the most abundant and easily found in this coastal plain, they are: *Ancistrus multispinis* (Regan, 1912) (Siluriformes: Loricariidae), *Kronichthys lacerta* (Nichols, 1919) (Siluriformes: Loricariidae), *Mimagoniates microlepis* (Steindachner, 1877) (Characiformes: Characidae), *Scleromystax barbatus* (Quoy & Gaimard, 1824) (Siluriformes: Callichthyidae), *Pareiorhaphis splendens* (Bizerril, 1995) (Siluriformes: Loricariidae) and an undefined species of *Rineloricaria* Bleeker, 1862 (Siluriformes: Loricariidae). Fishes were caught by electro-fishing or cast nets. Fishes were collected under the license number 10007 (Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio, Brazil).

All specimens of each fish species within each of the seven sub-basins were considered as belonging to the same panmictic population, because there are no obvious migration barriers within any of watersheds. Thus, data from fishes collected in various sampling sites within each sub-basin were pooled. Details of the sampling sites are found in Table 1.

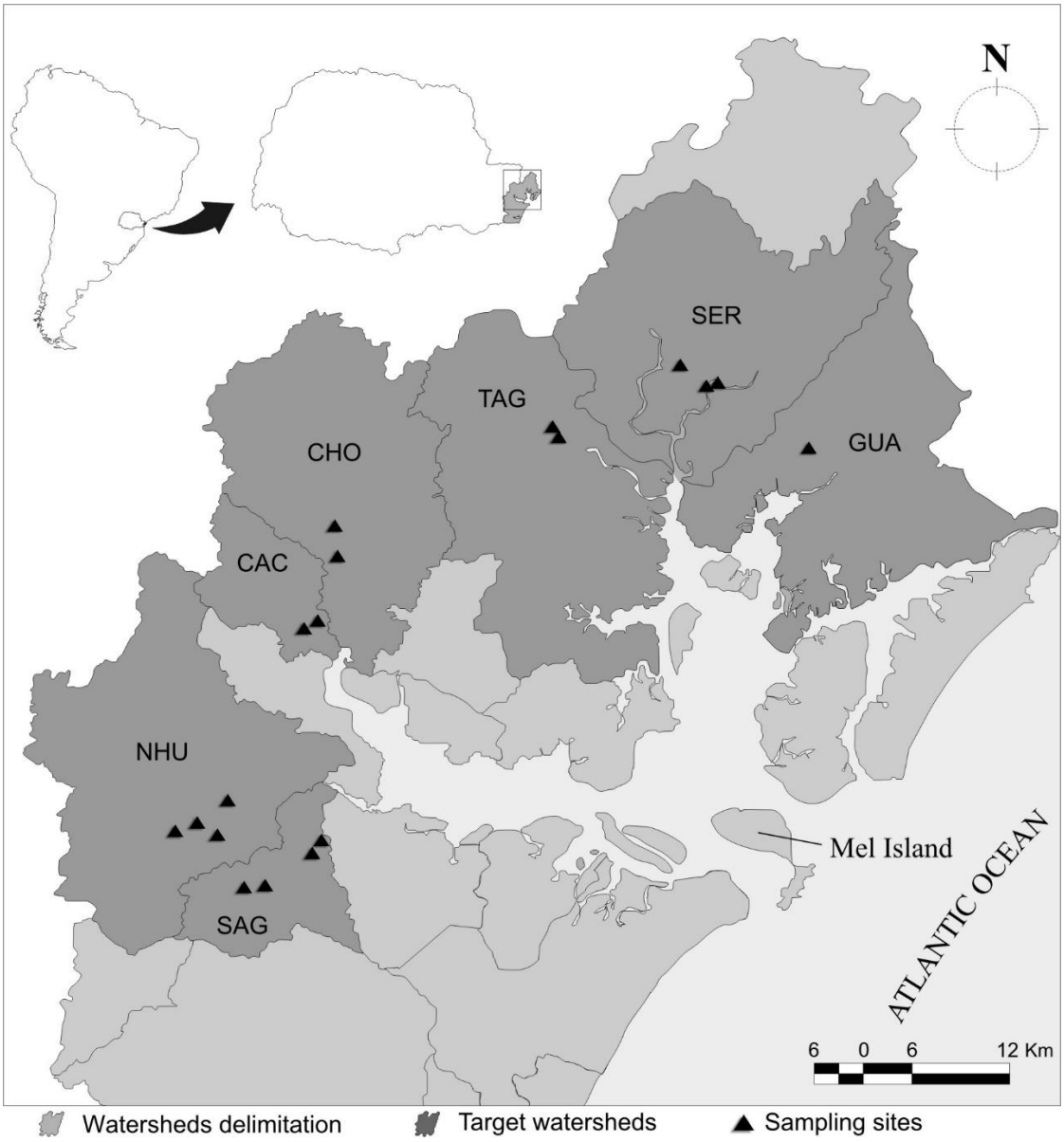


Figure 1. Sampling sites for *Ancistrus multispinis*, *Kronichthys lacerta*, *Mimagoniates microlepis*, *Pareiorhaphis splendens*, *Rineloricaria* sp., and *Scleromystac barbatus* in seven watersheds around the Paranaguá Bay, Paraná, Brazil. Watersheds names are abbreviated as: SAG) Sagrado; NHU) Nhundiaquara; CAC) Cacatu; CHO) Cachoeira; TAG) Tagaçaba; SER) Serra Negra; GUA) Guaraqueçaba.

Table 1. Details of sampling sites. Watersheds, rivers, geographical coordinates and municipality.

Site	Hidrographical sub-basins	River/stream	GPS coordinate	Locality
1	Sagrado	Sagrado	S 25° 33' 23.93" W 48° 48' 04.97"	Morretes, PR, Brazil
			S 25° 32' 14.29" W 48° 46' 00.08"	Morretes, PR, Brazil
			S 25° 31' 34.64" W 48° 45' 03.20"	Morretes, PR, Brazil
		do Meio	S 25° 33' 32.78" W 48° 48' 55.93"	Morretes, PR, Brazil
2	Nhundiaquara	do Pinto	S 25° 30' 50.30" W 48° 50' 34.27"	Morretes, PR, Brazil
		Marumbi	S 25° 30' 31.49" W 48° 52' 30.26"	Morretes, PR, Brazil
			S 25° 30' 11.15" W 48° 51' 25.38"	Morretes, PR, Brazil
			S 25° 29' 10.46" W 48° 49' 48.08"	Morretes, PR, Brazil
3	Cacatu	Cacatu	S 25° 19' 25.03" W 48° 45' 06.99"	Antonina, PR, Brazil
		do Nunes	S 25° 20' 19.23" W 48° 47' 04.60"	Antonina, PR, Brazil
4	Cachoerira	Mergulhão	S 25° 17' 37.85" W 48°44' 23.79"	Antonina, PR, Brazil
		Saci	S 25° 14' 13.92" W 48° 45' 09.92"	Antonina, PR, Brazil
5	Tagaçaba	Tagaçaba	S 25° 11' 38.74" W 48° 31' 17.81"	Antonina, PR, Brazil
		Capivari	S 25° 11' 03.42" W 48° 31' 22.82"	Antonina, PR, Brazil
6	Serra Negra	Pederneiras	S 25° 08' 34.83" W 48° 23' 38.08"	Guaraqueçaba, PR, Brazil
		Bananal	S 25° 10' 41.51" W 48° 22' 51.43"	Guaraqueçaba, PR, Brazil
			S 25° 10' 31.25" W 48° 22' 18.63"	Guaraqueçaba, PR, Brazil
7	Guaraqueçaba	Morato	S 25° 12' 48.87" W 48° 17' 52.62"	Guaraqueçaba, PR, Brazil

Molecular procedures

Pieces of muscle tissue were fixed in hypersaturated saline DMSO/EDTA solution (Seutin et al., 1991). The remaining parts of the fishes were fixed in 10% formalin, preserved in 95% ethanol, and deposited in the collection of Museu de História Natural do Capão da Imbuia, Prefeitura de Curiiba, Brasil.

Total genomic DNA was extracted following the standard protocols of EZ-DNA kit (Applied Biosystems) and ChargeSwitch® gDNA Tissue kit DNA iPrep™ (Invitrogen) through the iPrep™ robot. Fragments of mitochondrial genes, cytochrome b (CytB) and cytochrome oxidase subunit I (COI), and nuclear intron of the Adenosine Triphosphate Synthase subunit beta (ATPSβ) and nuclear Rhodopsin fragments (RHO) were amplified for the species using the primers specified in Table 2. Only CytB fragments could be amplified for *Ancistrus multispinis* and ATPSβ was amplified only for *Mimagoniates microlepis*. PCR amplifications were performed in a total volume of 25 µL containing 3 mM $MgCl_2$, 2.5 U Taq polymerase, 1x buffer, 0.4 mM of each dNTP, 2 nM of each primer, and 1 ng/µl genomic DNA template. PCR cycles consist of an initial denaturation

step of 4 min at 95 °C; followed by 35 cycles of 45 s at 94 °C, 45 s at 45 °C for CytB, 60 °C for COI, 51°C for RHO, and 52 °C for ATPSβ, the cycle ends with 45 s at 72 °C; and a final extension of 5 min at 72 °C. After detection of positive PCR products in agarose electrophoresis, the purification of successful DNA amplified was made with PEG 8000 (Amresco). DNA sequencing reactions were carried out in 10 µl reaction mixes consisting of: 0.2 µM of each primer, 0.5 µl Big DyeTM (Applied Biosystems), 0.1 x reaction buffer, and 5 ng/µl template DNA. The final product was purified using SephadexTM G-50 (GE Healthcare Bio-Sciences AB). Both strands of nucleotide sequences were edited in software Staden v.1.6.0 (Staden, 1996), and aligned using the ClustalW algorithm (Thompson et al., 1994) implemented in BioEdit v.7.0.5.2 (Hall, 1999). The haplotype sequences are find in GenBank.

Table 2. Primer sequences for amplification and sequencing used in the phylogenetic analysis.

Gene fragment	Primers	Sequence of primer 5' - 3'	Reference of Primer
cytochrome b (CytB)	GLUDG.L	TGACTTGAARAACCAYCGTTG	Palumbi (1996)
	H16460	CGAYCTTCGGATTACAAGACCG	Perdices & Doadrio (2001)
cytochrome oxidase subunit I (COI)	FF2d	TTCTCCACCAACCACAARGAYATYGG	Ivanova et al. (2007)
	FR1d	CACCTCAGGGTGTCCGAARAAYCARAA	Ivanova et al. (2007)
ATP synthase subunit beta (ATPSβ)	ATPSbf1	GTGAGGGHAAYGATTTHTACCATGAGATGAT	Jarman et al. (2002)
	ATPSbr1	CGGGCACGGGCRCCDGGNGGTTCGTTCAT	Jarman et al. (2002)
Rhodopsin (RHO)	RHPco1F	CATCGTCCGGAGTCCTTATG	Chen et al. (2003)
	RH1039R	TGCTTGTTCATGCAGATGTAGA	Chen et al. (2003)

Phylogenetic inferences

Bayesian Inference analyses were conducted in the software Beast v.1.7.5 (Drummond et al., 2012) to infer the phylogenetic history of lineages of individuals. Every fragment of gene available for a specific fish species was analyzed concatenated in Beast. Hence, it was implemented in four independent MCMC runs for 30 million generations sampled at each 1,000 trees. The suitable model of nucleotide substitution was determined in jModelTest v.0.1 (Posada, 2008) for each alignment of DNA fragment, assuming the corrected Akaike Information Criterion (AICc) (details in Table S1 in supplementary material). The independent runs were checked in Tracer v.1.5 (Rambaut & Drummond, 2009)

and combined using Log Combiner, and the maximum clade credibility tree were generated using Tree Annotator, both packages of BEAST. Phylogenies and posterior probabilities were calculated in strict molecular clock after a 10% burn-in. To provide an approximate time frame for the diversification histories of lineages we used the substitution rates of 0.0076 substitutions/site/million years for CytB (Zardoya & Doadrio, 1999). Mutation rates of the other fragments were estimated during independent runs in BEAST. Closely related species were chosen from Genbank (available in: http://www.ncbi.nlm.nih.gov/genbank/) to root the haplotype trees. For each respective genus, the outgroup was composed by *Ancistrus clementinae* Rendahl, 1937 and *Ancistrus macrophthalmus* (Pellegrin, 1912); *Kronichthys heylandi* (Boulenger, 1900) and *Kronichthys subteres Miranda Ribeiro, 1908*; *Mimagoniates inequalis* (Eigenmann, 1911) and *Mimagoniates lateralis* (Nichols, 1913)*; Pareiorhaphis azygolechis* (Pereira & Reis, 2002), *Pareiorhaphis steindachneri* (Miranda Ribeiro, 1918), and *Pareiorhaphis vestigipinnis* (Pereira & Reis, 1992)*; Rineloricaria jaraguensis* (Steindachner, 1909) and *Rineloricaria uracantha* (Kner, 1863)*; Scleromystax kronei* (Miranda-Ribeiro, 1907)*, Scleromystax macropterus* (Regan, 1913)*,* and *Scleromystax prionotos* (Nijssen & Isbrücker, 1980).

Each fish cladogram was used as input trees in the algorithm PACT, a substitute to Brooks Parsimony Analysis (BPA; Brooks, 1981, 1990; Brooks et al. 2001, Brooks & McLennan, 2001; 2002). PACT no more requires that the taxon–area cladograms be converted into matrices, *contra* BPA. PACT algorithm combines partial information from each of several taxonomic clades and allow us to reconstruct substantial parts of the coevolutionary record of life integrating information of phylogenetic relationships and from geographic history of inhabited areas. It has been shown superior to other methods of biogeographical analysis because it does not specify a priori costs, weights, or probabilities of particular biogeographical events (Wojcicki & Brooks, 2005). However, the algorithm was executed by hand, since there is no software developed for it, despite the suggestion of Arias et al. (2008) that a package for this algorithm is already available due to the equivalence with the approach of Page (1994).

The first step to start the application of the algorithm is to replace the original terminal label in cladograms (unique sequences of concatenated haplotypes, in this case) with the label of area(s) associated to each terminal unit. Next, it is necessary to choose the first phylogeny. According to Wojcicki & Brooks (2004; 2005), any tree can be selected at this point without changing the result of the final concatenated tree. We chose to begin the construction of final tree with the area cladogram of *Mimagoniates microlepis* since it was the largest tree in number of operational taxonomic units (haplotypes). Start with the most complex cladogram is considered the best and easiest way to obtain the final product (Wojcicki & Brooks, 2004; 2005). Here we use the same terminology adopted by the authors, which named the combination of trees as “template area cladogram” and the final concatenated tree as “general area cladogram (GAC)”.

The next step is to select any tree as the second area cladogram, or the second input tree, and compare the common areas and nodes to combine them into the template area cladogram. We need to note which terminal labels in the second tree occur in the template area cladogram and which do not occur. Each area associated with consistent terminal labels in both trees indicates a match with previous pattern and these are combined. Each terminal label that do not occur in both cladograms is a new element and it is attached to the template area cladogram at the node where it is connected with a clade that have the same terminal labels composition. The combination of common elements should occur as long as they are connected at the same node and the addition of novel elements to the template area cladogram should be at the node where they first appear.

Once the novel elements in the second area cladogram have been added to the template area cladogram we should look again if any of them could be combined as well. These steps of searching for common patterns and novel elements should be repeated for all available input trees, until all of them are incorporated into the GAC. More details and some tests of the protocol are available in Wojcicki & Brooks (2004; 2005).

Therefore, GAC represents the integration of all phylogenetic information from six taxon–area cladograms, without the modification of data or the

application of weights. This process is named 'assumption 0' and must be followed strictly-data modification is not acceptable. All information in each area cladogram must be used in the phylogeographic analysis and the relationship of areas demonstrated in the GAC should be compatible with the phylogenetic relationships depicted in each area cladogram used as input trees to produce the final cladogram (Wiley, 1986; 1988a; 1988b; Zandee & Roos, 1987). The molecular dating was incorporated into the algorithm in order to ensure a proper representation of the spatial data relationship – i.e. the time frame of nodes was respected in a logical and consistent application of cladograms incorporated in the template area cladogram.

Branches of the final area cladogram were mapped to reveal the geographical patterns and the evolutionary process associated to isolation or dispersal events. We follow the protocol proposed by Lieberman (2000, 2003a; 2003b) and complemented by Halas et al. (2005) that proposed these methodological elements to test Taxon Pulses. Lieberman (2000; 2003a; 2003b) is based on the phylogenetic character optimization of Fitch (1970; 1971) to evaluate the distributional states, rather than the ancestry of nucleotides. The optimization uses the two-pass parsimony method of Farris (1970). First, from the tips to the base of GAC and after predicting the ancestral areas of the nodes from the base back to the tips of GAC. This mapping protocol recognize, in area cladograms, which nodes are associated with dispersal (biotic expansion) and which ones are linked to vicariance (isolation events). Isolations events are postulated when the number of occupied areas in the reconstructed spatial distribution of subsequent nodes decreases (oldest to younger nodes). Whereas, dispersal or biotic expansion are postulated when the number of occupied areas in subsequent nodes increases. Lieberman (2000; 2003a; 2003b) and Halas et al. (2005) protocols were designed to search for evidences of general patterns, thus, distinguishing among general nodes the ones that are shared by more than one species.

The predicted dates of each event were plotted in a diagram. They were obtained by the average of ages of the nodes calculated using molecular clock for phylogenies of each species involved in that general node. The confidence

intervals of molecular-clock dating were also taken into consideration and were obtained by the intersection of the confidence intervals in each shared node.

The dynamic of diversification for each species was evaluated in curves of extant lineages accumulated through time. These curves were obtained in the statistical program R v.3.1.2, using the packages ape, laser, geiger, and diversiTree (Paradis et al., 2004; Rabosky, 2006; Harmon, et al. 2008; FitzJohn, 2012), R Develompent Core Team) with the individual phylogenies of species rooted by midpoint.

Regression analyses were performed to assess the relation of three independent variables over the total extant lineage-richness in each area. First, the amount of isolation/expansion events detected in each watershed and calculated by the reconstruction of ancestral areas. Second, drainage areas of each watershed obtained from Serviços Geográficos do Exército assembled in Paula et al. (2006) to the sub-basins of the Sagrado and Nhundiaquara rivers, and in Planos Locais de Desenvolvimento da Maricultura (2010) to the remaining sub-basins. Third, the average of volumetric flow rate for all basins, during eight consecutive months, were obtained from the Instituto de Águas do Paraná (Data available in Table S2 in supplementary material).

Paleodrainages reconstruction

The current underwater topographical relief can be used to infer connections from ancient rivers in the coast of Paraná State, predicting the past landscape exposed above water due to marine regressions in glacial periods. The inference of paleodrainages followed the methodology applied in recent studies for wider coastal areas (e.g. Dias et al., 2014; Thomaz et al., 2015). Ancient rivers (paleodrainagens) that existed during low sea-levels were inferred from the botton topography, modeled by the digital elevation model (DEM) GEBCO_08 at 30 arc-second resolution and based on bathymetric and topographical data (Available at http://www.gebco.net/). Paleodrainages were revealed in ArcGIS v.10.3.1 with surface and hydrological tools. Raster imperfections were corrected through the fill option and sink option to identify the gaps in DEM. From the corrected image we generated the flow direction and flow

accumulation of raster. Thus, the drainage system was reconstructed with the map algebra operator with a 100 pixels threshold. Finally, contour lines were added to show the relief based on nowadays depth.

Results

Difficulties in amplification resulted in variable number of fragments for each fish species. Fragments of approx. 1000 bp of the mtDNA CytB were sequenced for all species. Fragments of > 630 bp of mtDNA COI were used for *M. microlepis*, *K. lacerta*, *Rineloricaria* sp., and *P. splendens*. Nuclear fragments of approx. 800 bp of RHO gene were sequenced for *Kronichthys lacerta*, *Rineloricaria* sp., *S. barbatus*, and *P. splendens*. As a complement, a 390 bp of ATPSβ was sequenced for *M. microlepis*.

The compilation of the different fragments above generated different number of unique concatenated haplotype sequences for each species (the terminal units in a single input trees) (see table S1 in supplementary material). *Rineloricaria* sp. presented 51 unique sequences of haplotypes (post-concatenation) distributed in seven watersheds. *Kronichthys lacerta*, *S. barbatus* and *M. microlepis* showed 37, 47, and 64 concatenated haplotypes, respectively in six watersheds. *Ancistrus multispinis* exhibited 21 concatenated haplotypes distributed in five watersheds. *Pareiorhaphis splendens* presented 30 concatenated haplotypes distributed in four watersheds.

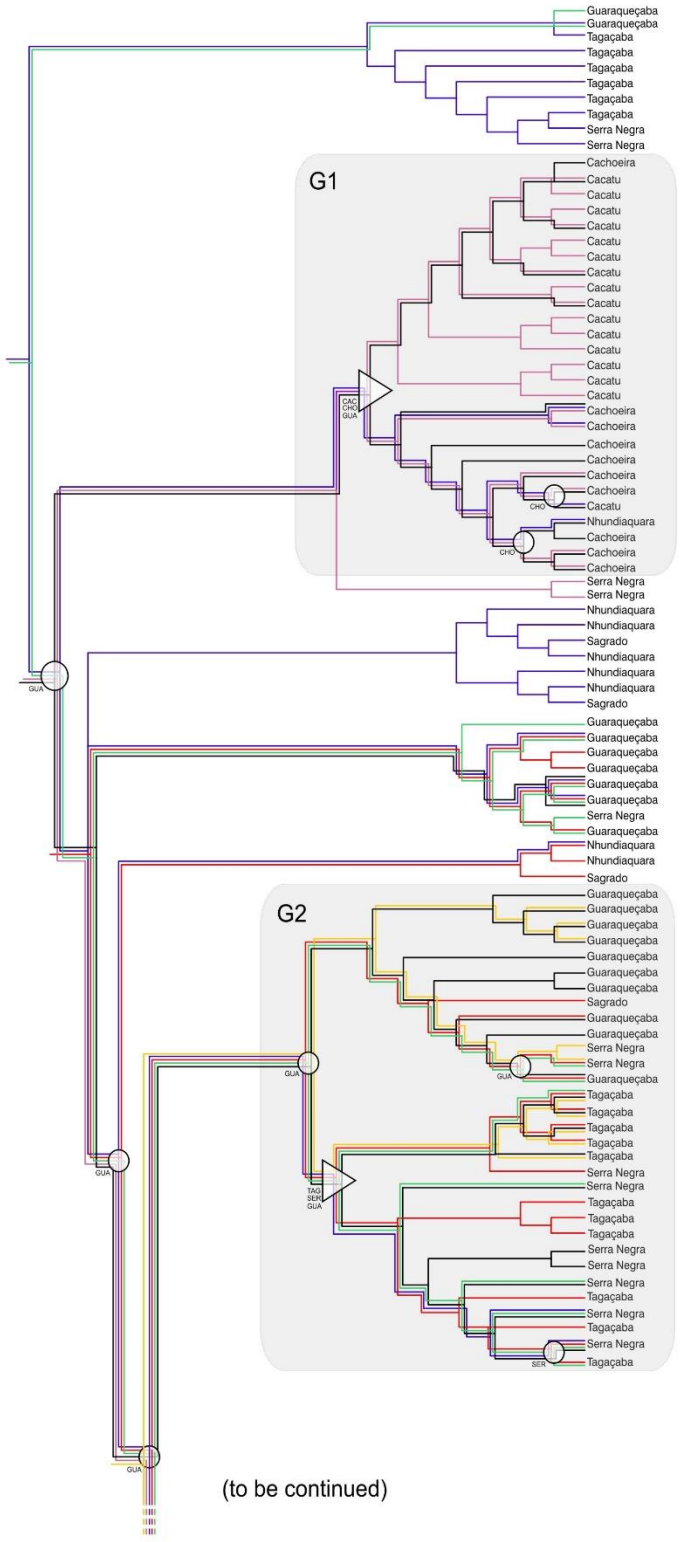
Posterior probability values greater than 65% are shown above the branches in all single phylogenies. Gray bars on the branches of each phylogeny show the 95% HPD interval of node ages. The oldest branches are present in *S. barbatus* and *Rineloricaria* sp., both from the Guaraqueçaba sub-basin (Fig. S1 in supplementary material). *Pareiorhaphis splendens* has the most limited spatial distribution of concatenated haplotypes, which are distributed almost exclusively in western sub-basins of Paranaguá estuary, with the exception of two concatenated haplotypes found in the Serra Negra sub-basin (Fig. S1A). However, *A. multispinis*, *S. barbatus*, and *K. lacerta* show a clear pattern of

segregation between western/northern watersheds around Paranaguá Bay. For these species, there are clades exclusively distributed in western watersheds (Sagrado, Nhundiaquara, Cacatu, aand Cachoeira sub-basins) and other clades with haplotypes exclusively distributed in northern watersheds (Tagaçaba, Serra Negra and Guaraqueçaba sub-basins) (Fig. S1B-D). On the other hand, the phylogenetic relationship of concatenated haplotypes in *Rineloricaria* sp. and *M. microlepis* show an apparently chaotic distribution of concatenated haplotypesis less geographically structured. These species present concatenated haplotypes found only in northern watersheds, but there are clades with a wide geographical distributions of lineages (Fig. S1E-F).

Phylogenetic analysis for comparing trees (PACT)

The final area cladogram present 169 terminal units, of which 66 are formed by the compilation of at least two species and did not violate the Assumption 0. The remaining 103 terminal units are linked to single clades (involving only one species) that do not share their geographic history with any other lineages according the PACT approach. The GAC comprises 155 nodes which 64 are shared nodes - 15 shared by two species, 22 by three species, 21 by four species, 5 represent five species, and 1 node composes the shared history of all six species. Nineteen nodes (30 %) are associated to events of isolation or biotic expansion, according to the mapping reconstruction of ancestral area in the GAC. Four nodes are associated to isolation events and fifteen are related to expansion events (Fig. 2).

Guaraqueçaba sub-basin was reconstructed as the ancestral area on basal nodes in the GAC (Fig. 2). Ancestral lineages of all six fish species were first introduced in this estuarine system from this northern watershed, but in distinct moments. *Rineloricaria* sp. and *S. barbatus* were present in the Guaraqueçaba sub-basin for more than one million years ago while *K. lacerta* and *P. splendens* colonized this same basin shortly thereafter associated to an expansion event. *Mimagoniates microlepis* represents an independent colonization and, later, A. *multispinis* was also introduced in association to another expansion event.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Tagaçaba
Tagaçaba
Serra Negra
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G1
Cachoeira
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Sagrado
G2
Guaraqueçaba
Guaraqueçaba
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Guaraqueçaba
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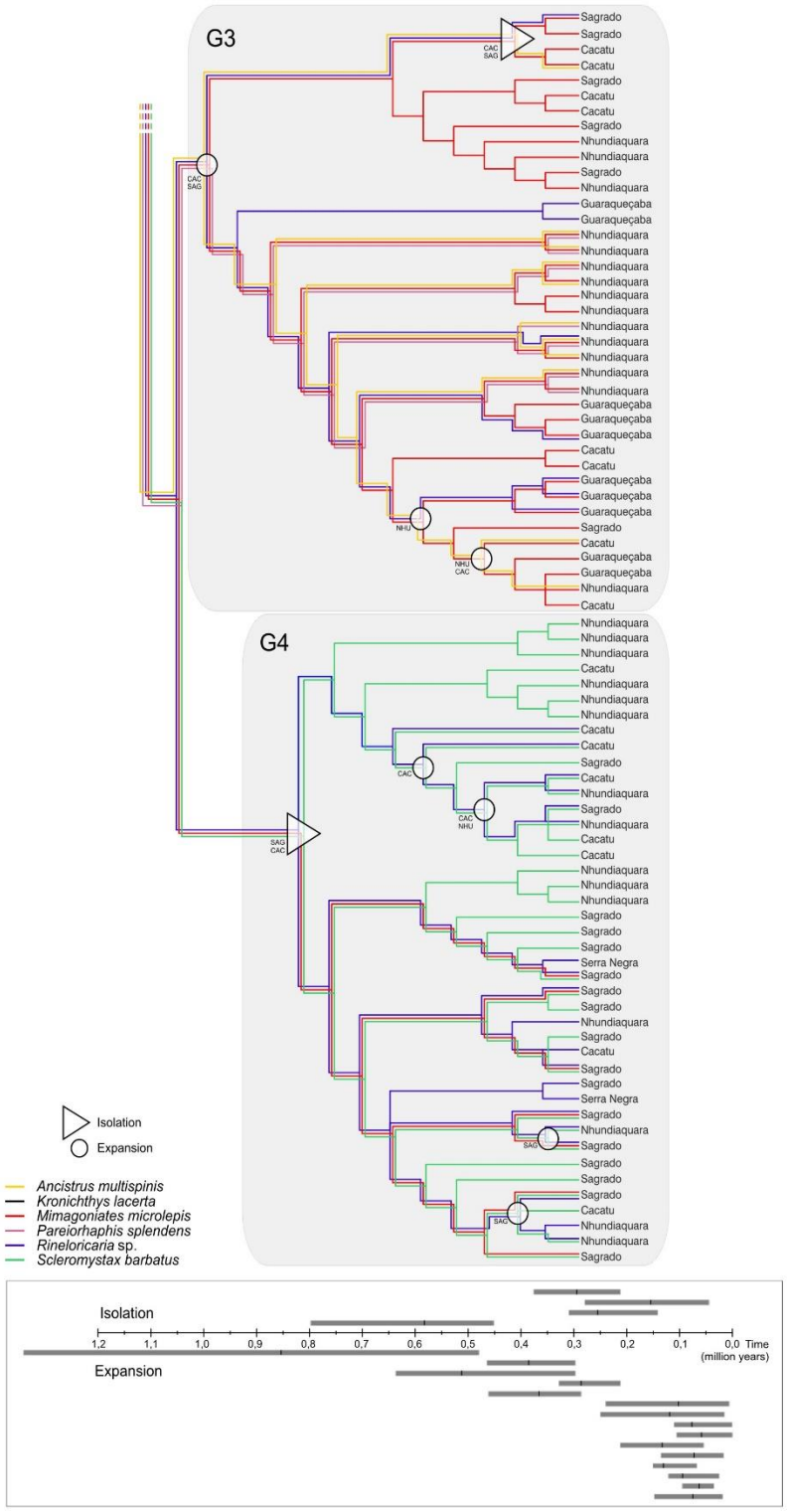

Figure 2. Final area cladogram from PACT of six freshwater fishes. Nodes resulting from isolation events are indicated in circles, expansion events are shown in triangles according Lieberman's protocol. All other nodes reflect unique events affecting only single clades. Abbreviation on nodes follow the names of watersheds: SAG) Sagrado; NHU) Nhundiaquara; CAC) Cacatu; CHO) Cachoeira; TAG) Tagaçaba; SER) Serra Negra; GUA) Guaraqueçaba.

The GAC was divided in four highlighted clades that encompass the majority of detected events by the reconstruction of shared nodes (Fig 2). G1 is basally associated with dispersion from Guaraqueçaba to the Cacatu and the Cachoeira sub-basins. Later in this clade, PACT reconstructed an isolation event between these watersheds and subsequent dispersions from Cachoeira sub-basin to the Cacatu and Nhundiaquara sub-basins. G2 indicated expansion among nearby northern sub-basins, a subsequent isolation between the Tagaçaba and the Serra Negra sub-basins and a recent dispersion from the Serra Negra to The Tagaçaba sub-basin. G3 and G4 have similar origin of lineages from Guaraqueçaba spread to Cacatu and Sagrado sub-basins. Both clades show two events of isolation between lineages of Sagrado and Cacatu sub-basins and then, dispersal events associated with terminal branches.

The average of event ages in the input trees and the intersections of the equivalent time of HPD intervals of shared nodes is shown in a time scale in the diagram on Figure 2. The 95% confidence interval was wide and covers a large period that encompasses more than one detected cycle of sea regression/transgression based on data of paleoclimatic reconstruction and paleo-sea-level indicators (Kowsmann & Costa, 1974; Correa, 1996; Angulo & Lessa, 1997; Suguio et al., 1985; Martin et al., 1996; Angulo et al., 2006). A precise definition about the age of each event is not possible and, thus, we cannot directly correlate these to known dating of marine regression or transgression in the region. However, we can identify an interpolated pattern of expansion and isolation. The fifteen expansion events are divided into three groups, one oldest expansion, another group with four biotic expansion events and a later group formed by the other ten expansion events associated with terminal branches. The four isolation events interpose these three groups of biotic expansions.

Curves of extant lineages accumulated through time exposed the diversification of fish lineages starting in ca. 500 kya. We observe periods of high accumulation of concatenated haplotypes interpolated with stationary phases for *Rineloricaria* sp. and *K. lacerta*, though this observation may be less accurate for other species (Fig. 3). The best example is *Rineloricaria* sp., in which there is a wave of increase in number of lineages at 300 kya that stops at 200 kya and

returns to increase at 170 kya. The accumulation lines also have different patterns of increase. The other four species, *A. multispinis*, *M. microlepis*, *P. splendens*, and *S. barbatus*, however, have duplicated the number of lineages in about one hundred thousand years.

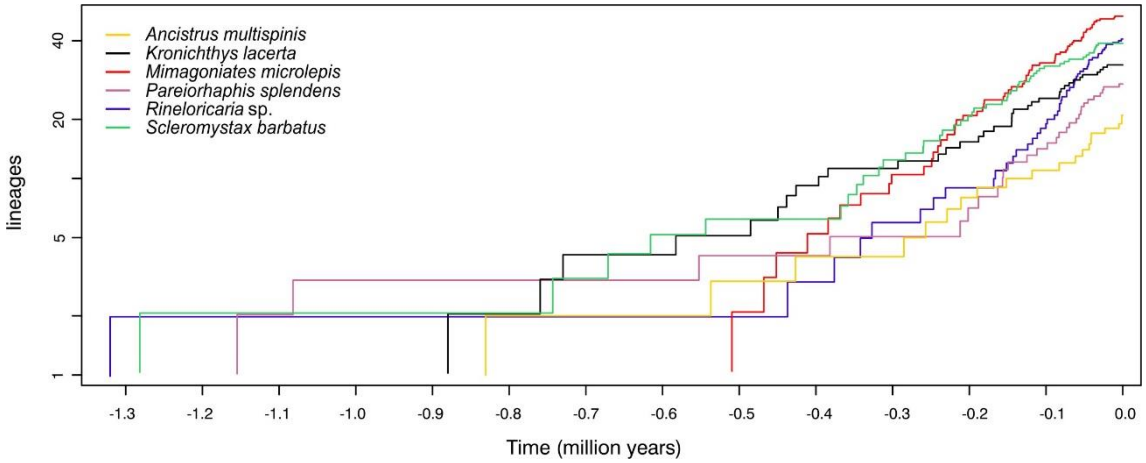

Figure 3. Logarithm curves of extant lineages accumulated through time of six freshwater fish species from seven watersheds in the coast of Paraná State, Brazil.

The drainage area and the volumetric flow rate did not show significant relation to the total richness of extant lineages. However, linear regression analysis indicated a positive relation between the richness of extant lineages in watersheds and the number of isolation/expansion events in which each sub-basin was involved ($r^2 = 0.711$, $p = 0.017$) (Fig. 4).

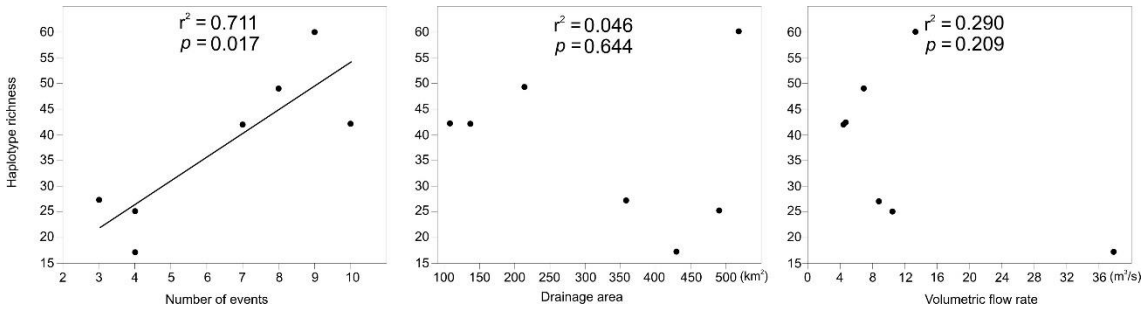

Figure 4. Linear regression between the number of extant lineages in each watershed versus the amount of isolation/expansion events, drainage area, and average of volumetric flow rate in each area.

Reconstruction of paleodrainages from topographic and bathymetric data predicted ancient rivers that probably were connected in the past during the last

lowest sea-levels. Paleodrainages are indicated as thick lines in Figure 5. There is an obvious relationship between the connectivity of submerged ancient rivers and the current distance of watersheds. Nearby watersheds are previously connected in a downstream direction, however, we observe a pattern of western connection linked to a northern channel that are connected before the Mel Island (see details in Figure 1) in an area with no more than 10 m deep.

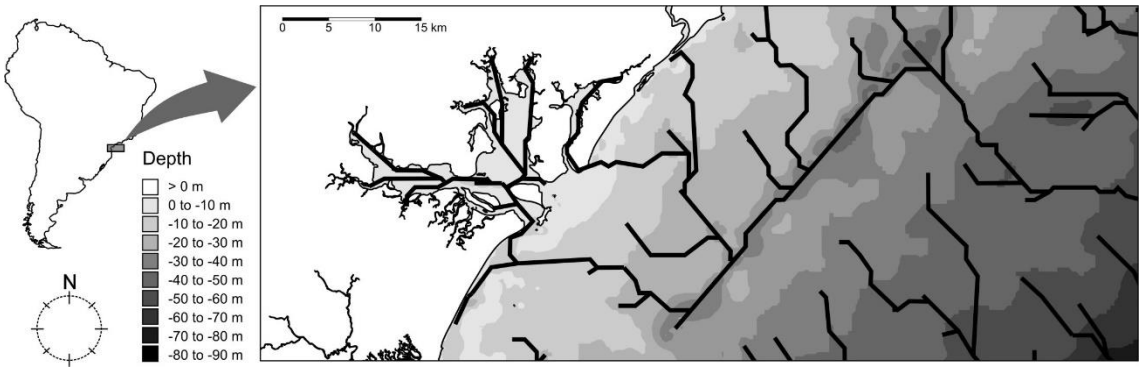

Figura 5. Map of paleodrainages in Paranaguá Bay, Paraná State, Brazil. Ancient channels are indicated by thick lines. Narrow line indicates the current shoreline. Gray areas represent -10 m deep.

Discussion

Freshwater fishes in drainages around Paranaguá Bay have been associated in a common history at least since the Pleistocene. The results, however, do not conform to a simple vicariant scenario. Instead, they exhibit a complex history including alternating generalized episodes of expansion and isolation incorporated in the Taxon Pulse hypothesis (Erwin, 1979; 1981; 1985) and many dispersion events not associated with generalized expansion events mentioned above. Diversification by taxon pulse is supported by cyclic processes of expansion and isolation of fish populations on coastal watersheds that we associate to cycles of variation of the sea-level.

This is the first time that the algorithm PACT was used to reconstruct patterns of diversification in a short time scale, without enough time to result in the origin of new species. The shared history of six freshwater fishes reveals an

oscillatory pattern (i.e. Taxon Pulse) representing a prevalent diversification dynamic in the watersheds studied.

The search for evidences of Taxon Pulse focusing on freshwater fish populations in a small geographic area is novel. Previously, only Doadrio & Domínguez (2004) and Domínguez-Domínguez et al. (2006) worked with freshwater fishes and used BPA and secondary BPA (Brooks, 1981; 1990; Brooks et al., 2001; Brooks & McLennan, 2001; 2002). Domínguez-Domínguez et al. (2006) indicated a historical biogeography of freshwater fishes in Central Mexico as result of a series of allopatric speciation and subsequent dispersal events. But, Halas et al. (2005) based on analysis of 33 clades of species inhabiting areas in Mexico were the first to propose an explicit protocol for discovering pulses of diversification and to provide a large scale picture of complex biotas assembled by taxon pulses.

PACT has been shown as a methodology that permits a more parsimonious search for ancestral areas compared to other computational analysis (Lim, 2008). By applying this algorithm, Halas & Winterbottom (2009) examined the reef biota, including eight clades of marine fishes in the region of Indo-Pacific Ocean, however they detected very little congruence between area relationships and the histories of the examined taxa. On the other hand, many recent studies with freshwater fishes worldwide demonstrate patterns that comply with the Taxon Pulse assumptions, although this ecological theory was not mentioned by the authors (eg. Day et al., 2013; Nevado et al., 2013; Pereira et al., 2013; Roxo et al., 2012; Roxo et al., 2014; Bruno et al., 2015; Thomaz et al., 2015). We suspect that these studies may agree on patterns of pulse diversification with oscillatory contractions and expansions of taxa. We hope they are reanalyzed with the PACT algorithm in the future.

The final area cladogram in this study is consistent with all input trees (Fig. S1); data were not removed nor modified, thus the assumption 0 was satisfied. Our analysis identified fifteen events of biotic expansion and four isolation events interpolated in the shared history of the six species of coastal fishes. Initially, using phylogenetic character optimization of areas mapped onto the GAC based on Lieberman (2000; 2003a; 2003b) and Halas et al. (2005) we identified the

lineage origin of each species commonly associated with the Guaraqueçaba sub-basin, but in different nodes and times. According to the reconstruction, *Rineloricaria* sp. and *S. barbatus* were the only species (among the six species studied) present in the watersheds of the Paranaguá system at more than one million years ago. Subsequently, *K. lacerta* and *P. splendens* colonized the Guaraqueçaba sub-basin apparently in association with an expansion event which likely occurred during low sea-level when extensive connections among coastal rivers occurred in an area now flooded by the salt water. *Mimagoniates microlepis* and *A. multispinis* were the last two to colonize initially the same Guaraqueçaba sub-basin. The introduction of *A. multispinis* is also linked to a biotic expansion event.

Multiple introductions occurred into this northern sub-basin at different times for each species and it is an important evidence that colonization of the current lineages of freshwater fishes around the Paranaguá Bay is strongly associated with dispersal of species derived, most likely, from coastal basins north of the Paranaguá Bay. The reconstruction of local paleodrainages supports to northern origins for the four species more recent in the bay. During sea-level retreat of approximately 50 m deep, our reconstruction of paleodrainages indicates that ancient rivers were connected with northern drainages rather than to southern river systems (Fig. 5).

Indeed, Thomaz et al. (2015) also reconstructed paleodrainages of the Paranaguá Bay linked to northern paleodrainages and found an exceedingly high genetic differentiation between populations of *Hollandichthys multifasciatus* (Eigenmann & Norris, 1900) from the Paranaguá estuary and southern populations from another bay geographically close. Moreover, haplogroups of *Hoplias malabaricus* (Bloch, 1794) from farther north watersheds in the Brazilian coastline, and isolated by the Serra do Mar mountains, are also phylogenetic closer to populations from Paranaguá Bay (Pereira et al., 2013).

All singular nodes in the GAC (Fig. 2) are additional unique events of dispersal/expansion and reflect species-specific events of dispersal or sympatric diversification of haplotypes. At first glance, they are not interesting since they do not reflect the shared history of two or more species, but they are inherent to the

particular biology of each species and may represent stochastic events occurring in the region. According to Brooks (2005) and Halas et al. (2005), the absence of particular clades in some areas is better explained by the lack of specific participation in that particular expansion episode, rather than an extinction post-dispersal. The absence of clades due to extinction has the potential to obscure paleobiogeographic patterns because a region that experienced differential extinction will simply show a distinct pattern through time (Lieberman, 2000; 2002b). However, we argue that the absence of particular clades in some areas subsequent to isolation events may be fundamentally the result of lineage extinctions due to effects of genetic drift. This becomes more evident by recognizing that during high sea-level, the extension of most rivers that comprise the present watersheds in the system of the Paranaguá Bay were significantly reduced against the foothills of the Serra do Mar. With the expected reduction in the population of the freshwater fishes isolated in these river fragments and with the reduction of genetic variability, bottlenecks are expected.

We acknowledge that the inclusion of events involving only one species in the reconstruction of ancestral area can obscure the pulses by over-emphasizing these unique events and can generate spurious results. So, evaluating each node in each phylogeny separately, is not as robust as assessing nodes in a GAC and evaluate each single node in a second-phase evaluation, after the assessment through shared nodes. Patterns that were not apparent from phylogenetic analysis alone could be revealed by analyses that summarize phylogenetic data from multiple clades simultaneously. So, it makes historical biogeographic methods that include multiple clades in a temporal framework more powerful than single clade analyses or non-calibrated analyses (Folinsbee & Evans, 2012).

Therefore, our results are better depicted in Figure 6 and described below. A dispersal from Guaraqueçaba sub-basin to the Cachoeira and Cacatu sub-basins was the first event detected (Fig. 6a) involving *Rineloricaria* sp. *K. lacerta* and *P. splendens*, which we related as the result of sea-level regression and the connection among freshwater bodies in composed watersheds presently submerged. In the second stage (Fig. 6b), an isolation event of lineages of *K. lacerta* and *P. splendens* from the neighboring Cacatu and Cachoeira sub-basins,

which we related to a marine transgression. Then, the third stage (Fig. 6c) showed four dispersal events sequentially involving all species, which we related to a single marine regression event and responsible for the largest colonization of the coastal plain with lineages reaching all sub-basins. Three of these dispersions are originated from the Guaraqueçaba sub-basin. The first one to Nhundiaquara sub-basin, the second spread to the neighboring Serra Negra and Tagaçaba sub-basins, and the last one to the Sagrado and the Cacatu sub-basins. Next, a new set of dispersion events, most likely associated to a new cycle of marine transgression, representing the fourth stage (Fig. 6d), broke the connection between the rivers and isolated populations of five species, even from nearby watersheds such as theTagaçaba/Serra Negra and the Cacatu/Sagrado.

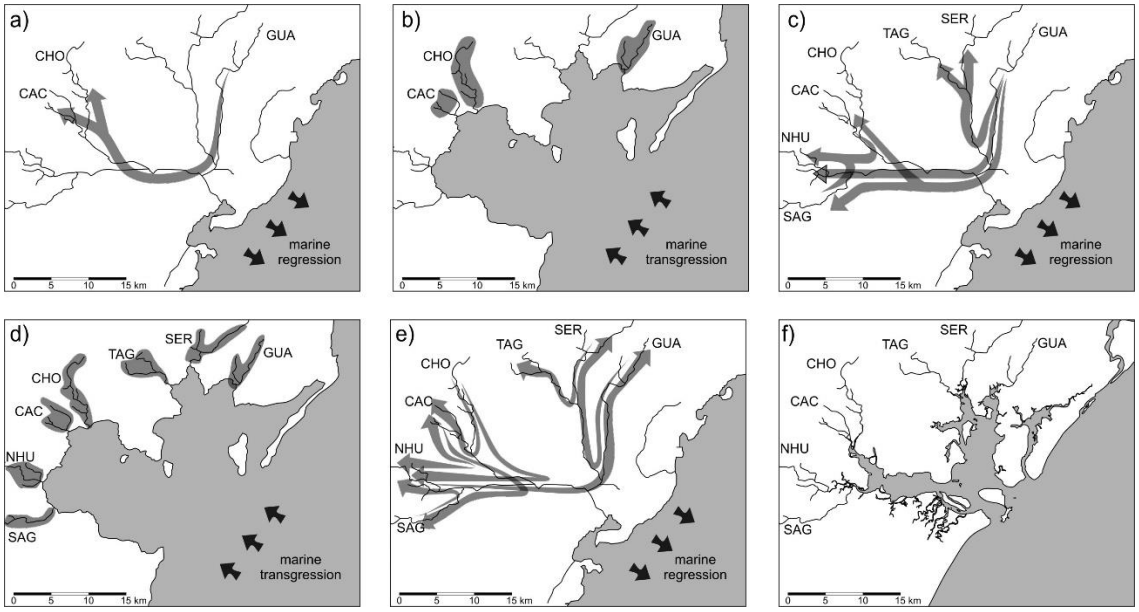

Figura 6. Diagram of historical isolation and expansion events of freshwater fish lineages in Paranaguá Bay, Brazil. Letters a to f represent stages of transgression and regression of the sea since the past until today.

Finally, the fifth stage (Fig. 6e) shows the latest events detected in the GAC. There are ten dispersal events. In clade G1, two dispersions are from the Cachoeira sub-basin in which lineages arrived in Nhundiaquara and Cacatu sub-basins. In G2 a dispersion from Guaraqueçaba to the Serra Negra sub-basin and another one from Serra Negra to the Tagaçaba sub-basin. In G3 a single expansion event from the western sub-basin to northern areas was reconstructed, from the Nhundiaquara to the Guaraqueçaba sub-basin; another

dispersion from Nhundiaquara to the nearby Cacatu sub-basin was also revealed. Finally, in G4 there are four dispersions related to nearby sub-basins, two of them from Sagrado to the Nhundiaquara sub-basin, other in the reverse way and the last one from Cacatu to the Nhundiaquara sub-basin.

The frame below on Figure 2 exposes these events together in a temporal scale and the average ages of each event can better revealed by the interpolated patterns. However, the last ten dispersal events are somewhat unexpected, if the proposed Taxon Pulse scenario is accepted. Based in the current reconstruction, it should be expected that the latest events detected close to apical units in the GAC are related to isolations, which match to the current high sea-level isolating freshwater population in the smaller components of each watershed, compatible with the reconstructed marine transgression at 123 kya (Angulo & Lessa, 1997; Suguio et al., 1985; Martin et al., 1996). However, we have two interpretations. First, the time of fixing genetic differences could be insufficient to detect the isolation events in this population-level approach. Nevertheless, the distribution of haplotypes and *Fst* values based on allelic frequency estimated for three of these six fish species in the same watersheds provide evidence of isolation and the influence of the last sea-level transgression (Tschá et al. unpublished). Second, what we may be seeing are signals of dispersal assisted by extensive freshwater plumes in the river mouth. Biotic expansion of fishes could have been related to connections during extreme rain periods involving neighboring watersheds (e.g. Chakona et al., 2013a; 2013b; Tschá et al., unpublished). This seems a plausible argument since eight of the ten nodes are related to neighboring basins.

A framework which enables the combination of calibrated phylogenetic data to test evolutionary hypotheses is the most effective manner to correlate geographical events. However, since the new proposal of PACT, only two manuscripts tried to incorporate some kind of temporal calibration in their data. Lim (2008) used two calibration points in the phylogenies of eight genera of New World bats to estimate the timing of speciation. Folinsbee & Evans (2012) temporally calibrate GACs *a posteriori* using fossil date and molecular clock estimates from previously published historical biogeographical analyses.

Folinsbee & Evans (2012) place a temporal context in an intuitive method that may indicate uncertainties from pseudo-congruences in the GAC, flagging potentially problematic data and indicating a need to review the input trees. They also suggest that dating the nodes using the average ages associated with the nodes, as in this study, is one way by which data can be summarized on GAC.

However, we detected some methodological limitations in the present study. First, the branch support values in some clades might weaken the historical relationship of areas suggested in each input tree. Nevertheless, the uncertainties caused by the weak supported clades would not significantly change the final configuration of GAC. For example, the low supported relationship between concatenated haplotypes did not affect the display of areas, because most clades remained with the same match in the GAC and recovered the same ancestral reconstruction. Thus, we accept that the trees generated in BEAST were the best available configuration for the implementation of PACT algorithm. Second, the lack of known specific mutation rates and inaccuracy of dating events of speciation hinder to a correct temporal reconstruction. The molecular clock did not generate precise dating, which complicates the temporal association between the diversification of lineages and the known dating of sea-level changes in the region. Therefore, this study is limited to relate the reconstructed patterns of diversification with the ages of events associated to the shared nodes. We understand that limitations of molecular clock are common in population studies with recent time approaches (e.g. Grant, 2015).

An oscillatory pattern of diversification is also supported by the curves of extant lineages accumulated through time if it evidences phases of higher accumulation of new lineages and more stationary phases, similar to waves of diversification. However, we observed such “waves” of diversification only for *Rineloricaria* sp. and *K. lacerta* (Fig. 3). These two species did not show an exponential or linear increase of lineages accumulation but periods of acceleration in the accumulation of new lineages between periods of relative stasis. The accumulation lines of both species seem to display comparable episodes of explosive emergence of new lineages. However, the other four species did not present the same configuration. we have however, be careful with

artifactual results that may be generated by inconsistency in evaluating these data only by eye.

Regression analyses points to a positive relation of extant lineage-richness only against the times in which they were involved in isolation/expansion events. However, no relation could be established between the number of extant lineages in the watersheds and the drainage area or volumetric flow rate in each watershed. These results suggest that cyclic evolutionary events of expansion and isolation are preponderant in promoting diversification of lineages in the region than local spatial features. Nevertheless, a mountainous terrain might act as ecological restrictions and may be associated to the absence of intermediate haplotypes in northern sub-basins (see Tschá et al. unpublished). We also suspect that physiographic features, such as the presence of waterfalls and rapid streams in northern sub-basins, can be related to the predominant dispersal flow direction towards the western basins. Only once dispersal events were reconstructed from west to north, all other fourteen dispersal events involved mainly neighboring sub-basins or were from north to west (Fig. 6).

The last 500 thousand years were crucial to the current lineages diversification and composition. During this period few river captures have been documented and it is not directly related to the evolutionary history in our study. River captures between coastal and inland sub-basins were described in the south-eastern drainages of Brazil, but pre-date the Pleistocene in opposition to our data (e.g. Malabarba, 1998; Ribeiro, 2006).

We understand that sedimentation and erosion processes often cause noise in paleodrainages reconstruction obtained by bathymetric data (Conti, 2009). However, the reconstruction of paleodrainages based on available bathymetric data is the best tool to reveal ancient rivers in submerged reliefs. We notice that the sea-level retreat of 10 m deep would be enough to connect all current rivers in the coast and allow the contact between previously isolated populations of freshwater (Fig. 5). Therefore, recent isolation events may be relatively brief compared to the longer periods of low sea-level and connectivity during the Pleistocene (see Shackleton, 1987; Siddall et al., 2003; Miller et al., 2011). Shorter cycles of rise and retreat of the sea-level, in relatively faster

timeframes than the glacial-interglacial periods, may also have played a significant role in the diversification of ichthyofauna along the estuary of Paranaguá.

In conclusion, this study reveals a complex history of the fish populations analyzed, but suggests the strong influence of sea-level changes in the pulse diversification of coastal freshwater fishes. Repeated episodes of transgression and regression of the ocean offered opportunities for population isolation/contraction and expansion in multiple phylogenetically unrelated clades. Taxon pulse has proved to be an applicable model also in reduced spatial scale. We suggest the algorithm PACT as an essential tool for analyses even in population-level and capable of revealing oscillatory patterns of dispersal and isolation even with insufficient time for allopatric speciation.

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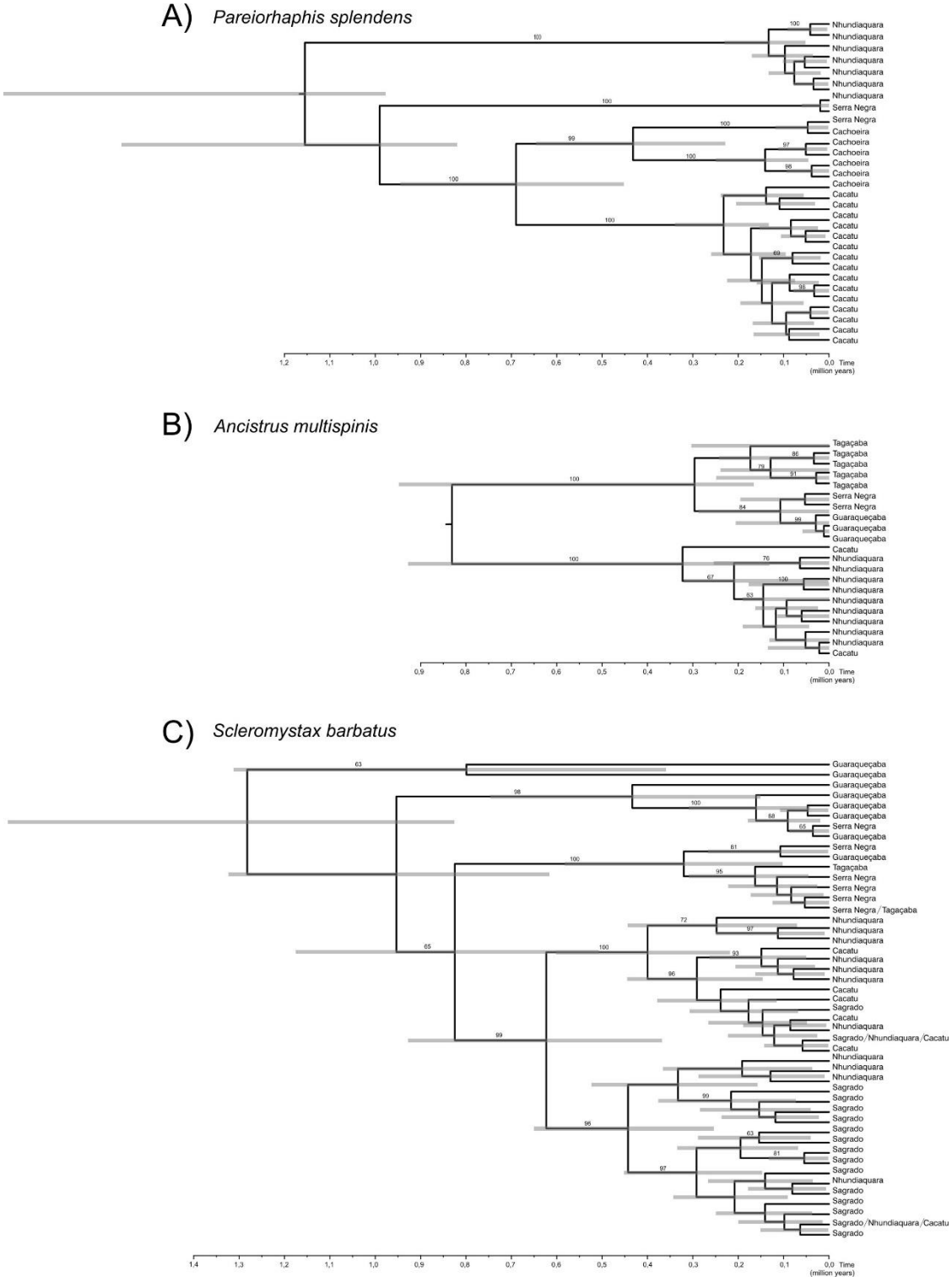
Supplementary material

Table S1. Details of substitution model used per species for each fragment. Haplotype number (*h*), sample size (n) and total number of concatenated haplotypes for each species.

	DNA fragment	Substitution model	Sagrado *h*	Nhundiaquara *h*	Cacatu *h*	Cachoeira *h*	Tagaçaba *h*	Serra Negra *h*	Guaraqueçaba *h*	TOTAL *h*	n	Concatenated haplotypes
Ancistrus multispinis	CytB	HKY + G	x	9	2	x	3	2	3	21	24	21
Kronichthys lacerta	CytB	TN	x	2	3	6	3	2	5	20	60	37
	COI	HKY	x	1	3	2	1	3	3	11		
	RHO	TN + G	x	1	2	2	1	3	6	6		
Mimagoniates microlepis	CytB	TN + G	10	15	4	x	8	2	10	43	173	64
	COI	HKY + G	8	9	4	x	4	1	10	29		
	ATPSβ	HKY	1	1	3	x	3	2	2	7		
Pareiorhaphis splendens	CytB	TN + G	x	5	14	6	x	1	x	26	39	30
	COI	TN	x	3	3	2	x	1	x	9		
	RHO	HKY	x	2	3	1	x	1	x	5		
Rineloricaria sp.	CytB	TN + G	11	14	6	1	5	7	6	41	207	51
	COI	TN + G	3	5	3	1	3	3	6	17		
	RHO	HKY + G	1	5	1	3	3	2	3	8		
Scleromystax barbatus	CytB	TN + G	15	14	6	x	2	5	8	47	185	47
	RHO	HKY	4	3	2	x	1	1	2	7		

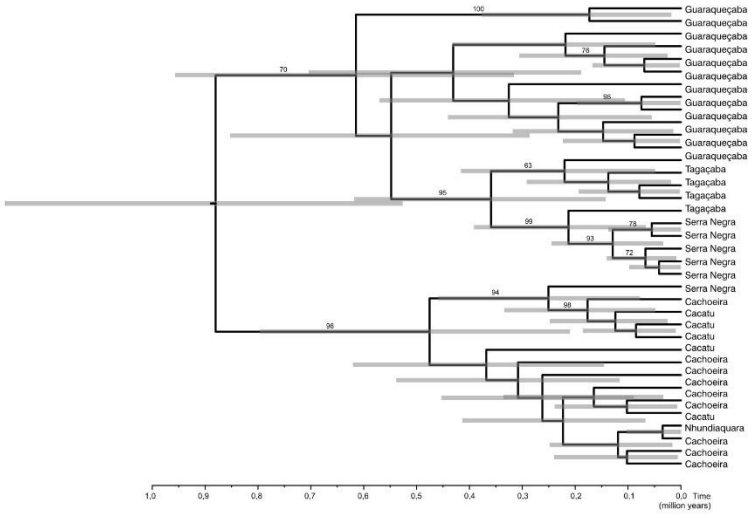
Table S2. Total drainage area and average of volumetric flow rate values used to assess the linear regression against the number of lineages in each watershed.

Watershed	Drainage area (km^2)	volumetric flow rate (m^3/s)
Sagrado	137.70	4.60
Nhundiaquara	519.30	13.31
Cacatu	107.46	4.66
Cachoerira	429.18	37.80
Tagaçaba	359.15	8.84
Serra Negra	490.71	10.45
Guaraqueçaba	214.53	6.92

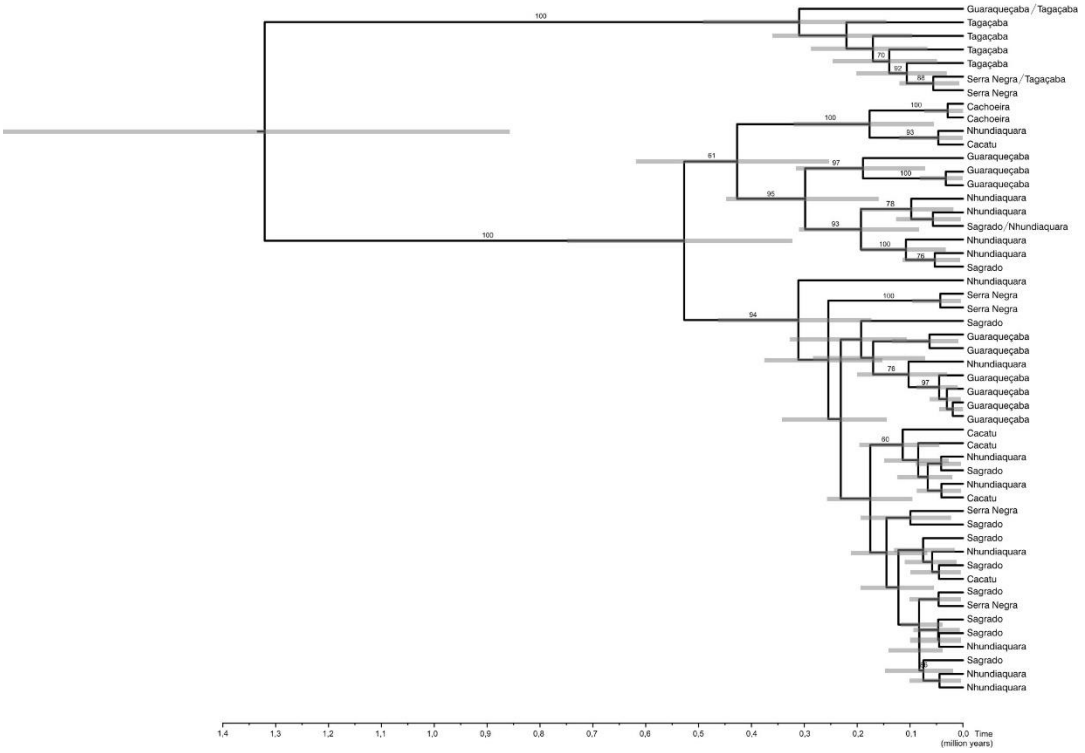


(to be continued)

D) *Kronichthys lacerta*



E) *Rineloricaria* cf. *jaraguensis*



F) *Mimagoniates microlepis*

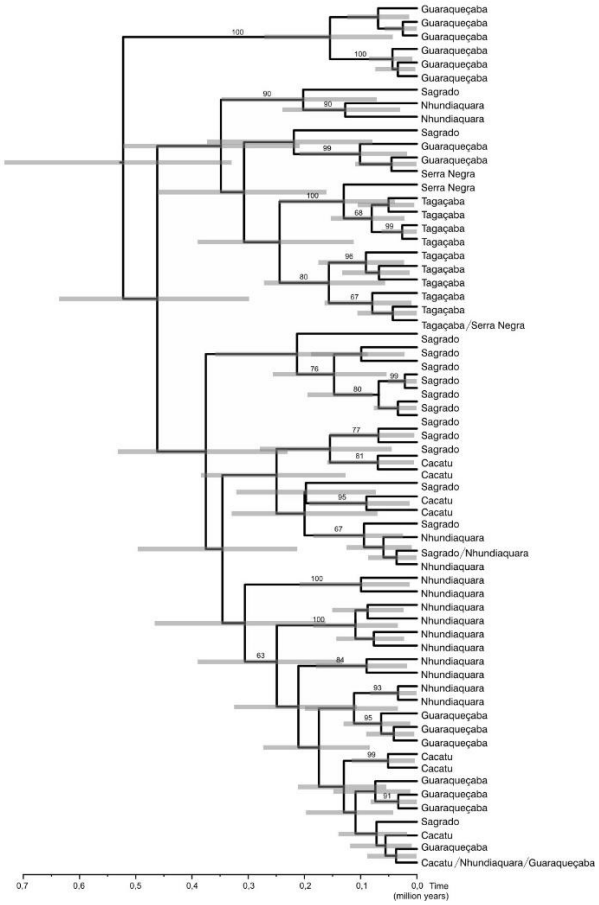


Figura S1. Input trees of the six freshwater fish species converted in area cladograms according to the haplotype distribution. Gray bars are node ages with 95% HPD. Support values above branches are posterior probabilities.

CONSIDERAÇÕES FINAIS

Uma das espécies envolvidas nessa pesquisa foi aqui apresentada como *Rineloricaria* sp. Porém, através de fragmentos do gene citocromo c oxidase subunidade I e do banco de dados no Barcode of Life Data System (acessível em: http://www.boldsystems.org/), a espécie apresenta-se filogeneticamente próxima a *Rineloricaria langei* Ingenito, Ghazzi, Duboc & Abilhoa, 2008; *Rineloricaria kronei* (Miranda Ribeiro, 1911) e *Rineloricaria jaraguensis* (Steindachner, 1909), todas coletadas em bacias hidrográficas geograficamente próximas. No entanto, a espécie desse estudo difere morfologicamente de qualquer uma das três espécies de *Rineloricaria* citadas anteriormente, pois possui seis séries de placas abdominais laterais, seguindo a nomenclatura taxonômica proposta por Schaefer (1997). A resolução taxonômica e filogeográfica desse grupo é ainda pouco esclarecida na região e diagnoses futuras são necessárias para averiguação da identidade taxonômica da espécie em questão.

A presente tese sustenta a ideia de que mudanças no nível do mar contribuíram para a diversidade genética da ictiofauna dulcícola em um sistema costeiro complexo, desde o Pleistoceno. Foi possível observar um distinto padrão na reconstrução da história demográfica de espécies litorâneas comparada a espécies de bacias adjacentes em relação aos Planaltos de Altitude Paranaenses. Também foram obtidas evidências de diferenciação genética acumulada em populações de peixes de água doce isoladas pela água do mar em bacias costeiras. Foi também revelado inédito padrão populacional associado a variações do nível do mar e que se encaixam nos pressupostos da hipótese de *Taxon Pulse* (1979; 1981; 1985). Transgressões e regressões marinhas são compatíveis com os eventos de isolamento e expansão detectados para as populações de peixes na planície costeira paranaense.

Combinar dados filogenéticos e de distribuição dos táxons em um contexto de biogeografia histórica para gerar hipóteses evolutivas é a maneira mais eficaz de identificar eventos correlacionados (Brooks & McLennan, 2002). Testar a hipótese de *Taxon Pulse* através do algoritmo PACT (Wojcicki & Brooks,

2004; 2005) torna-se o passo primordial para aceitar que a história dos peixes costeiros é gerada por um processo de pulsos de diversificação, bem como primordial para elaboração de estratégias de conservação preocupadas com a dinâmica evolutiva (veja Erwin, 1991). O estuário da Baía de Paranaguá faz parte do Bosque Atlântico Brasileiro, uma província da sub-região Paranaense (Morrone, 2001) e um remanescente da Floresta Atlântica. Esta área ainda com um bom grau de preservação é um sistema dinâmico de barreiras naturais com uma história evolutiva ímpar para a fauna dulcícola. A compreensão aqui obtida de sua influência na estrutura genética de comunidades costeiras foi essencial para se ter um panorama local dos mecanismos que fizeram dessa área tão característica. O método aqui empregado pode ser também aplicado a outras regiões onde processos subsequentes de isolamento e reconexão ocorreram em resposta a alterações do nível oceânico, como por exemplo na Bacia Amazônica.

Termos como museu e berçário evolutivo são propostos para drenagens costeiras e terras baixas da América do Sul (e.g. Hubert & Renno, 2006; Hubert et al., 2007; Bloom & Lovejoy, 2011; Roxo et al., 2014). Em uma visão macroevolutiva, museus definem regiões de maior estabilidade ou baixa perturbação ambiental que atuariam como refúgios na preservação de ramos filogeneticamente antigos, onde taxas de extinção são baixas em comparação a combinação das taxas de especiação e imigração (Stenseth, 1984). Berçário evolutivo, por outro lado, designa áreas onde taxas de especiação excedem as taxas de extinção resultando em um aumento da riqueza de espécies e permitem a dispersão para áreas adjacentes (Stebbins, 1974). Roxo et al. (2014) sugerem que drenagens costeiras do Atlântico serviram tanto de berçário evolutivo para diversificação inicial de loricarídeos quanto de museu para muitas linhagens desses cascudos que permaneceram confinadas em bacias costeiras da região. Todavia, em uma visão microevolutiva, a diversificação das linhagens de peixes de água doce é um balanço dinâmico de diferenciação, extinção e expansão biótica dessas linhagens para bacias hidrográficas adjacentes. A associação com movimentos eustáticos desde o Pleistoceno, no entanto, torna a planície costeira instável. Por mais que áreas de cabeceiras de rios atuem como microrefúgios durante incursões marinhas promovendo estruturação genética de

metapopulações, esse cenário cíclico parece mais relacionado ao termo berçário evolutivo, atuante no aumento global da diversidade genética do litoral paranaense.

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